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REMARKS

Claims 1-8 are currently pending and stand rejected. Claims 1-2 and 5-7 are amended and claims 9-12 are added herein. Support for the present amendments can be found throughout the specification and claims as originally filed. Support for the present amendments to claims 1 and 2 can be found, for example, at page 1, lines 8-17; page 4, lines 16-21; etc. Support for the present amendments to claims 5 and 6 can be found, for example, at page 1, lines 8-17; page 4, lines 16-27; page 6, line 23 through page 7, line 17; and Figure 2. Support for the present amendment to claim 7 can be found, for example, at page 4, lines 25-27; page 6, line 23 through page 7, line 17; Figure 2; and original claim 7. Support for new claims 9 and 10 can be located in original claims 1 and 5, respectively. Support for claims 11 and 12 can be found, for example, in the specification at page 4, line 27. No new matter is added and entry is respectfully requested.

Objections to the Sequence Listing

The sequence listing was objected to for the recitation of the amino acid Asparagine at position 71 of $hPTH_{1-84}$. The Office requested amendment of the sequence listing to recite Aspartic acid at position 71 of PTH_{1-84} as indicated in Figure 1 of the present application. The Applicant thanks the Office for the careful review of the sequence listing, as filed. Accordingly, the Applicant includes herewith a corrected CRF of the sequence listing in conformance with the Office's request.

Objections to the Specification

The specification was objected to by the Office for supposedly containing an "incomprehensible statement" regarding the description of the set of peptides encompassed by the term "CIP." The Office has required clarification of the description of "CIP" to provide a *comprehensible* description of the intended meaning of this term. Although the Applicant asserts that the meaning of the term "CIP" is apparent in the present specification, the specification is amended herein to provide a more elaborate, albeit redundant, explanation of this term.

For example, the intended meaning of the term "CIP" in the specification comprises a contiguous portion of human PTH having an amino acid sequence set forth in SEQ ID NO:5 (PTH_{1-84}), wherein the CIP has the following characteristics: a) the N-terminal amino acid residue of the CIP starts at any position spanning from position 2 through position 34 of the PTH_{1-84}; and b) the C-

terminal amino acid residue of the CIP ends at position 84 of the PTH_{1-84}. This description includes and refers to a set of peptides, such as PTH_{2-84}, PTH_{3-84}, PTH_{4-84}, PTH_{5-84}, PTH_{6-84}, PTH_{7-84}, PTH_{8-84}, PTH_{9-84}, PTH_{10-84} . . . PTH_{28-84}, PTH_{29-84}, PTH_{30-84}, PTH_{31-84}, PTH_{32-84}, PTH_{33-84}, up to and including PTH_{34-84}. In essence, the phrase objected to by the Office is intended to mean the same thing as if Applicant had listed out each individual CIP peptide between the listed boundaries.

Accordingly, the Applicant herein amends the specification to more particularly spell out the intended meaning of the disputed term in a less truncated form. No new matter is provided and entry is respectfully requested.

The Office has further objected to the specification for the improper recitation of the phrase "SEQ ID NO:". The Applicant thanks the Office for the careful reading of the specification and herein amends the specification to correct these typographical errors. Entry is respectfully requested.

In addition, the Office has objected to the specification for multiple apparently miscited sequences. Again, the Applicant thanks the Office for the careful reading of the specification and herein amend the specification to correct these typographical errors. Entry is respectfully requested.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1-8 stand rejected under 35 U.S.C. § 112, second paragraph as purportedly indefinite. Similar to the objections to the specification described above, the Office indicates that the recitation of the description of the term "CIP" in claims 1, 2, 5 and 6 is indefinite. Claims 1, 2, 5 and 6 are amended herein to more particularly spell out the intended meaning of the term "CIP." As this further description of this term is considered redundant, no new matter is added. It is believed that the presently described amendments to claims 1, 2, 5 and 6 merely clarify certain aspects of the present invention and, as these amendments are not made for reasons related to patentability, they do not narrow the intended scope of the claims.

Claims 1 and 5 are further rejected as purportedly indefinite for reciting the limitation "therapeutically effective," as it is unclear to the Office what this term means. In response, the Applicant respectfully directs the Office's attention to the phrase following the limitation "therapeutically effective" in claim 1. Therein the claim indicates a "therapeutically effective, but non-toxic amount that reduces the occurrence of hypercalcemia or osteosarcoma in the patient

resulting from the administration of CAP." This underlined portion of claim 1 indicates one aspect of the intended meaning of the disputed claim limitation, and this purpose conforms with the overall purpose of present claim 1, *i.e.*, to reduce the occurrence of hypercalcemia or osteosarcoma in a patient that is receiving (or has received) administration of CAP. Claim 5 is amended herein to remove the phrase objected to by the Office. Accordingly, withdrawal of this rejection is respectfully requested.

Claim 5 is also rejected for the recitation of the phrase "the administration of CAP." Claim 5 is amended herein to remove this phrase. Moreover, claim 5 is amended to address the Office's concern regarding osteosarcoma and hypercalcemia. Accordingly, withdrawal of this rejection is respectfully requested.

Claim 7 is rejected for the inclusion of an additional method step, purportedly without indicating when the step should be carried out and for what effect. Accordingly, claim 7 is amended herein to specifically indicate that an additional step is contemplated in this claim and that such step is undertaken to monitor treatment. As such, withdrawal of this rejection is respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-8 stand rejected under 35 U.S.C. § 112, first paragraph as purportedly not enabled. The Office specifically indicates that the examples and experimental results provided in the specification are insufficient to support claims to "*a method for treating osteoporosis* using a PTH antagonist (or CIP), wherein a patient is also being treated with a PTH agonist (or CAP)." Office Action page 4. Moreover, the Office indicates that "it is not a general practice to apply a combination of an agonist and an antagonist of the same receptor for the treatment of a disease in most clinical scenarios, as each would neutralize the effect of the other." *Id.*

The claims are amended herein to be directed to either (1) a method for reducing the occurrence of hypercalcemia or osteosarcoma in a patient that has received or is being administered cyclase activating parathyroid hormone (CAP) or analogues thereof; or (2) inducing the CAP rebound effect in a patient. Moreover, only claims 1-4 are limited to a method wherein the patient is being administered, or has received administration of CAP or analogues thereof. Claims 5-8 encompass the co-administration or previous administration of CAP in patients, but are not

specifically limited thereto. Finally, CAP and CIP do not target the same receptor. For example, CAP acts through the 1-84 PTH/PTHrp receptor. And, it has been recently demonstrated that PTH_{7-84} (an exemplary CIP composition) effects internalization, and ultimately leads to a down regulation, of the 1-84 PTH/PTHrp receptor without concomitant activation. *See* Sneddon, W.B. et al., *J. Biol. Chem.* (2003) 278(44):43787-96 (attached as **Exhibit A**). In addition, PTH_{7-84} (an exemplary CIP composition), in its role as a competitive antagonist of CAP, generally binds and activates a C-terminal receptor independent of the PTH/PTHrp receptor. *See* Divieti, P. et al., *Endocrinology* (2002) 143(1):171-6 (attached as **Exhibit B**).

Normal bone is maintained and strengthened through a balance of bone resorption and bone rebuilding. Patients afflicted with osteoporosis generally have a higher bone resorption rate (*i.e.*, characterized by increased osteoclast activity) than bone rebuilding rate. Thus, the treatment for osteoporosis traditionally consists of the inhibition of bone resorption. CAP administration to a patient afflicted with osteoporosis will often lead to increased serum calcium concentrations and, occasionally, hypercalcemia and/or osteosarcoma. *See* FORTEO® product insert (attached as **Exhibit C**). CAP operates through the PTH/PTHrp receptor and accelerates bone turnover. In contrast, CIP operates through a C terminal PTH receptor and decelerates bone turnover. As is evident in light of Divieti, P. *et al.* (2002) (cited above), PTH_{7-84} (an exemplary CIP) acts to inhibit bone resorption by inhibiting osteoclast formation. *See id.*; Divieti, P. *et al.*, J. *Bone Miner Res Suppl* (2001) 1, S307 (attached as **Exhibit D**); Faugere, M.C. *et al.*, *J Am Soc Nephrol* (2001) 12: 764A (attached as **Exhibit E**).

In a patient, as CAP is administered, CIP production is stimulated via a biofeedback mechanism. Calcium homeostasis is maintained by the biological actions of CAP and CIP. As indicated above, CAP increases bone turnover and CIP decreases bone turnover. These two hormones play a significant role in the net bone turnover which, in turn, controls the serum calcium levels. Therefore, when a CAP based therapeutic composition is injected (or otherwise introduced) into a patient, the patient's body responds by increasing bone turnover with an associated rise in serum calcium levels. However, although not bound by theory, in order to bring both the turnover and the serum calcium level back under control, the body manufactures CIP in response to the CAP intervention. This biofeedback mechanism is similar to the way the CAP/CIP ratio changes in

response to an increase in calcium levels. As indicated in Faugere, M.C. *et al.*, *Kidney Int.* (2001) 60:1460-1468 (attached as **Exhibit F**), PTH_{7-84} levels increase relative to PTH_{1-84} levels in response to an increase in circulating calcium levels, thus reducing the ratio of PTH_{1-84} / PTH_{7-84}.

The present methods encompass an associated theory that CAP production *in vivo* can be affected by CIP administration. As indicated in the specification, the CAP rebound effect is believed to be the body's response to CIP administration; upon administration and increasing concentrations of CIP, parathyroid gland cells secrete CAP in an effort to return the CAP/CIP ratio to homeostasis with the pre-CIP administration levels. *See* the specification at page 6, lines 23-29. This CAP production is produced *in vivo* thus decreasing the occurrence of the potential, detrimental effects of exogenous CAP administration (*e.g.*, osteosarcoma and/or hypercalcemia). *See id.* at lines 29-30.

As the specification indicates and the Office acknowledges in the Applicant's experimental results, CAP administration led to an increase in serum calcium levels, CIP administration led to a decrease in serum calcium levels, and the combined administration of CAP with CIP led to calcium levels that stayed substantially the same (and slightly increased) in experimental animals over the course of two hours. *See* the specification at pages 6-7; Figure 2. Notably, the serum calcium levels in the control animals decreased over time. *See id.* Prior to testing, all experimental animals (including the control animals) had their parathyroid glands removed, thus prohibiting the production of CAP or CIP *in vivo*. Accordingly, if CIP acted solely by blocking the receptor of CAP, the serum calcium levels in animals that received the combination of CAP and CIP would fall at about the same level as the control animals. Interestingly, the Office acknowledges that this was not the case. Moreover, with regard to the duration of the experiment, two hours was sufficient time to witness detectible, if not dramatic, changes in serum calcium concentrations. As indicated above, these serum calcium concentrations are indicative of the rate of bone turnover.

The Office argues that an equimolar administration of CAP and CIP "would cause no significant net change in serum calcium levels, and as such, it is highly likely that such a combination would cause no significant net change in other biological effect." However, as CAP and CIP act on different receptors and CIP further acts to down regulate the 1-84 PTH/PTHrp

receptor (see above), it is likely that this assertion is not accurate (*see also* the discussion in the above paragraph regarding the Example set out in the present application).

The present methods are directed, in part, to returning the patient to a normal (or pre-osteoporosis) rate of bone turnover. As indicated above, bone turnover involves both bone resorption and bone formation, and certain disorders including osteoporosis are characterized by a higher resorption rate than bone rebuilding rate. Administering CIP to a patient receiving administration of CAP acts to stabilize serum calcium levels. Such stabilization reduces the occurrence of hypercalcemia and/or osteosarcoma. Moreover, this serum calcium level stabilization positively affects the CAP/CIP ratio and provides for a balance of the bone resorption rate versus the bone rebuilding rate. Accordingly, the present methods would be understood by one of skill in the art as effective at treating a patient that is receiving administration of CAP.

Administration of CIP to a patient that is not currently receiving CAP administration or to a patient that had previously received CAP administration also leads to the stabilization of serum calcium levels. Although not bound by theory, the CAP rebound effect is effected through the administration of CIP, which leads to a return of the CAP/CIP ratio to homeostasis and attendant balance of the bone resorption rate versus the bone rebuilding rate. Accordingly, the present methods would be understood by one of skill in the art as effective at inducing the CAP rebound effect in a patient, or reducing the occurrence of hypercalcemia or osteosarcoma in a patient that has received administration of, or is being administered, CAP or analogues thereof.

Based on the foregoing, the Applicant respectfully asserts that the present claims are enabled as written.

As to any issue of undue experimentation, it should be noted that optimization of dosage and administration are matters of routine experimentation once the active ingredient is identified, as it has been here. *See also* the specification at page 6, lines 16-20. The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *See In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976); *In re Wands*, 858 F.2d 731, 736-37, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988); *and* MPEP § 2164.01.

Listing of Exhibits

A. Sneddon, W.B. et al., *J. Biol. Chem.* (2003) 278(44):43787-96

B. Divieti, P. et al., *Endocrinology* (2002) 143(1):171-6

C. FORTEO® product insert

D. Divieti, P. *et al.*, J. *Bone Miner Res Suppl* (2001) Sept; 16: Suppl. 1, S-307

E. Faugere, M.C. *et al.*, *J Am Soc Nephrol* (2001) 12: 764A

F. Faugere, M.C. *et al.*, *Kidney Int.* (2001) 60:1460-1468

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no.532212000200. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: April 14, 2004

Respectfully submitted,

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Activation-independent Parathyroid Hormone Receptor Internalization Is Regulated by NHERF1 (EBP50)*

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Parathyroid hormone (PTH) regulates extracellular calcium homeostasis through the type 1 PTH receptor (PTH1R) expressed in kidney and bone. The PTH1R undergoes β-arrestin/dynamin-mediated endocytosis in response to the biologically active forms of PTH, PTH-(1–34), and PTH-(1–84). We now show that amino-truncated forms of PTH that do not activate the PTH1R nonetheless induce PTH1R internalization in a cell-specific pattern. Activation-independent PTH1R endocytosis proceeds through a distinct arrestin-independent mechanism that is operative in cells lacking the adaptor protein Na/H exchange regulatory factor 1 (NHERF1) (ezrin-binding protein 50). Using a combination of radioligand binding experiments and quantitative, live cell confocal microscopy of fluorescently tagged PTH1Rs, we show that in kidney distal tubule cells and rat osteosarcoma cells, which lack NHERF1, the synthetic antagonist PTH-(7–34) and naturally circulating PTH-(7–84) induce internalization of PTH1R in a β-arrestin-independent but dynamin-dependent manner. Expression of NHERF1 in these cells inhibited antagonist-induced endocytosis. Conversely, expression of dominant-negative forms of NHERF1 conferred internalization sensitivity to PTH-(7–34) in cells expressing NHERF1. Mutation of the PTH1R PDZ-binding motif abrogated interaction of the receptor with NHERF1. These mutated receptors were fully functional but were now internalized in response to PTH-(7–34) even in NHERF1-expressing cells. Removing the NHERF1 ERM domain or inhibiting actin polymerization allowed otherwise inactive ligands to internalize the PTH1R. These results demonstrate that NHERF1 acts as a molecular switch that legislates the conditional efficacy of PTH fragments. Distinct endocytic pathways are determined by NHERF1 that are operative for the PTH1R in kidney and bone cells.

Extracellular calcium homeostasis in vertebrate animals is primarily under the endocrine control of the parathyroid hormone (PTH)[1]/type I PTH receptor (PTH1R). The PTH1R, predominantly expressed in kidney and bone cells, belongs to class B of the large superfamily of G protein-coupled receptors (GPCRs) that consists of receptors for peptide hormones and neuropeptides (1). Class B GPCRs are characterized by a common topology and by their ability to couple to multiple signaling pathways via distinct G proteins.

PTH is synthesized by the parathyroid glands as a mature peptide of 84 amino acids that is stored in secretory vesicles and dense core granules. Reductions of extracellular calcium levels are detected by the calcium-sensing receptor on parathyroid chief cells and promote the release of PTH, which acts on bone (to increase resorption) and kidney (to augment reabsorption), thereby restoring serum calcium levels. PTH-(1–84) is usually the major form of PTH secreted by the parathyroid glands. However, recent analyses reveal that PTH fragments that are likely to be PTH-(7–84) are also secreted by the parathyroid glands and generated by peripheral metabolism (2, 3). These PTH fragments or their synthetic analogs are thought to be inactive on the PTH1R because, despite binding to the receptor, they fail to promote activation of the classical effectors adenylyl cyclase and phospholipase C (4–7). In fact, NH_2-terminally truncated PTH fragments behave as competitive PTH antagonists (8).

As with most GPCRs, the responses of the PTH1R to agonists are regulated by multiple mechanisms, including a well characterized and highly conserved process involving receptor phosphorylation by G protein-coupled receptor kinase 2 (9, 10) and arrestin recruitment (11–13). These processes contribute directly to PTH1R desensitization by facilitating the uncoupling of the receptor from its cognate G proteins, G_s and G_q. Following desensitization, the PTH1R is endocytosed into intracellular compartments, from which it can be either recycled to the membrane, leading to receptor resensitization

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‖ Recipient of a CIHR doctoral award.

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[1] The abbreviations used are: PTH, parathyroid hormone; PTH1R, type 1 PTH and PTH-related peptide receptor; hPTH1R, human PTH1R; GPCR, G protein-coupled receptor; EBP50, ezrin-binding protein 50; NHERF1, Na/H exchange regulatory factor 1; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DCT, distal convoluted tubule; PCT, proximal convoluted tubule; SaOS, human osteosarcoma cells; ROS, rat osteosarcoma cells; HA, hemagglutinin.

(14), or targeted for degradation, leading to receptor down-regulation (15, 16).

Increasing evidence demonstrates that PTH1R activation and endocytosis can be dissociated, with each event requiring distinct and specific receptor conformational states. PTH peptide analogs that efficiently activate the PTH1R but fail to induce arrestin-mediated internalization have been described (17). Conversely, PTH1R mutants have been generated that exhibit impaired ability to transduce G protein-mediated signaling but are phosphorylated by G protein-coupled receptor kinase 2 and internalized in response to PTH-(1–34) (18). These observations raise the possibility that PTH analogs that are unable to activate the PTH1R may be capable of inducing receptor endocytosis. In the present work, we tested this hypothesis. We now show that amino-terminally truncated PTH peptides internalize the PTH1R without antecedent or concomitant activation. We uncovered the molecular mechanism underlying this novel phenomenon and found that it occurs in a cell-specific manner that depends on the expression of the scaffolding protein EBP50, also known as NHERF1.[2]

NHERF1 is a cytoplasmic adaptor protein that contains tandem PDZ domains that have been implicated in protein targeting and in the assembly of protein complexes (19, 20). NHERF1 also possesses an ERM domain, which binds to the actin-associated proteins, ezrin, radixin, moesin, and merlin (21). NHERF1 recruits various cellular receptors, ion transporters, and other proteins to the plasma membrane of epithelia and other cells (22). Recently, Mahon and co-workers (23) reported that NHERF1 and -2 bind to the PTH1R through a COOH-terminal PDZ recognition motif and determined a role for NHERF2 in PTH signaling. Our findings demonstrate a novel action for NHERF1 as a molecular switch that determines the conditional efficacy of PTH fragments in bone and kidney cells. Additionally, the results establish the existence of distinct endocytic pathways for the PTH1R in response to either agonists or nonactivating PTH fragments. As such, they provide a new cellular mechanism for the regulation of GPCR function.

EXPERIMENTAL PROCEDURES

Cell Culture—The preparation, subcloning, characterization, and culture conditions for kidney and bone cells were as described (24, 25). Kidney cells were grown in a 50:50 mix of Dulbecco's modified Eagle's medium/Hamm's F-12 (10-092-CV; Mediatech, Inc., Herndon, VA), supplemented with 5% heat-inactivated fetal calf serum (Invitrogen) and 1% PSN (5 mg of penicillin, 5 mg of streptomycin, and 10 mg of neomycin/ml; Invitrogen). SaOS2 cells were grown in RPMI supplemented with 10% fetal calf serum. HEK-293 and ROS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections of HEK-293 cells grown to 75–90% confluence were performed using FuGENE 6™ (Roche Applied Science) according to the manufacturer's instructions. Empty pcDNA3 vector was added to keep constant the total DNA amount added per plate. Cells grown on 100-mm (DCT) and 60-mm (ROS) plates were transfected using 6 μg of total DNA. DCT and ROS cells stably expressing NHERF1 were generated by transiently transfecting NHERF1 cDNA in pcDNA 3.1 Hygro using FuGENE 6™. After 48 h, transiently transfected cells were trypsinized and plated on 150-mm dishes containing culture medium supplemented with 300 μg/ml hygromycin (Invitrogen) to select stable transfectants.

Complementary DNA Constructs—pEGFP-N2 plasmid encoding a full-length human PTH1R carboxyl-terminal EGFP fusion protein (PTH1R/C-EGFP) was kindly provided by C. Silve (INSERM, Paris, France). The PTH1R with EGFP introduced in the E2 extracellular domain (PTH1R/N-EGFP) has been previously described (26).

Mutation of the terminal amino acid of PTH1R, in PTH1R/C-EGFP, from methionine to alanine (M593A) was performed by PCR using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The fidelity of the construct was confirmed by sequencing (ABI PRISM 377; Applied Biosystems, Foster City, CA) and subsequent sequence alignment (NCBI BLAST) with human PTH1R (GenBank™ accession number XM 002895).

Cyclic AMP and Inositol Phosphate Assays—Ligand-stimulated accumulation of cAMP was determined in the presence of 1 μM 3-isobutyl-1-methylxanthine. Phosphatidylinositol hydrolysis was determined in the presence of 10 mM LiCl. cAMP and total inositol phosphates were measured chromatographically as previously described (17).

Receptor Internalization—PTH1R internalization was measured by quantitative, live cell confocal fluorescence microscopy in cells stably transfected with the hPTH1R-EGFP. Cells were plated on poly-D-lysine-coated 25-mm glass coverslips and analyzed at room temperature by confocal microscopy (Amersham Biosciences) equipped with a 488-nm argon/krypton laser. Emitted fluorescence was detected with a 515–540-nm band pass filter. PTH1R/EGFP internalization was analyzed by selecting the entire plasma membrane through a plane normal to and ~2–3 μm above the basal membrane surface (ImageScan; Amersham Biosciences). Sequential images were acquired at 1-min intervals. After obtaining three control images, the indicated ligand was introduced, and images were obtained for an additional 15–30 min to ensure that internalization was complete with any given maneuver. Internalization of PTH1R/EGFP was reflected by a loss of plasma membrane fluorescence, quantified as changes in pixel intensity. Fluorescence intensity was digitized at 16-bit resolution and converted to 256 grayscale levels for each image. The product of the number of pixels within the defined membrane area and the average pixel intensity was calculated for each time point. Kinetic parameters were determined by fitting the data to a sigmoidal nonlinear equation, where PTH1R internalization = bottom + (top − bottom)/(1 + $10^{(\log EC_{50} - \log[PTH])}$) and plotted using Prism (GraphPad Software, Inc.). Results are presented as the mean ± S.E. for the indicated number of independent observations.

Radioligand Binding and Internalization—Cells (100,000–200,000) prepared as described above were incubated on ice for 2 h with ~100,000 cpm of high pressure liquid chromatography-purified [^{125}I][Nle^{8,18},Tyr^{34}]PTH-(1–34)NH$_2$ in 250 μl of Dulbecco's modified Eagle's medium/F-12 medium containing 5% fetal bovine serum, essentially as described (27, 28). In brief, cells grown to confluence in 24-well plates were incubated for 2 h at room temperature (to achieve equilibrium binding). Under these conditions, the concentration of radioligand was ~0.1 nM. Following incubation, the cells were washed twice with ice-cold phosphate-buffered saline and collected in 0.5 ml of 0.1 N NaOH, and bound [^{125}I]PTH was assessed by γ spectrometry. Ligand internalization was measured as follows. Cells (100,000–200,000) were washed twice with ice-cold phosphate-buffered saline and incubated in 0.5 ml of Dulbecco's modified Eagle's medium/F-12 medium containing 5% fetal bovine serum at room temperature. At the indicated time points, surface-bound ligand was extracted by two 5-min incubations on ice with 50 mM glycine buffer (pH 3.0) containing 0.1 M NaCl. After acid extraction, the remaining cell-associated (internalized) radioligand was collected in 0.5 ml of 0.1 N NaOH. The amount of radioligand in each fraction was assessed by γ spectrometry. Radioligand internalization is expressed as the ratio (percentage) of internalized fraction over the total cell-associated ligand (surface plus internalized). Nonspecific binding and internalization were measured in parallel experiments carried out in the presence of 1 μM unlabeled PTH-(1–34). Curves were fit using a four-point logistic algorithm (Prism, GraphPad Software, San Diego, CA).

Arrestin Translocation—DCT cells grown on 100-mm dishes were transiently transfected with 1 μg of β-arrestin-2/GFP and 5 μg of hPTH1R-pcDNA3 (courtesy of Dr. Marc Caron, Duke University). After 48 h, the cells were split onto collagen-coated 25-mm coverslips. Arrestin translocation in response to PTH ligands was assessed at room temperature using real time live cell confocal microscopy as reported (29).

Dynamin Dependence—DCT cells were split onto 25-mm coverslips and transiently transfected with 1 μg of PTH1R/EGFP in the presence of 1 μg of K44A-dynamin-pcDNA3.1 (Dr. Orson Moe, University of Texas, Dallas) or empty pcDNA3.1 vector. PTH1R internalization in response to PTH ligands was then measured and quantified as outlined previously.

Coimmunoprecipitation—Six-well plates of HEK-293 cells were transfected with the different combinations of DNA constructs as indicated. Forty-eight h after transfection, the cells were rinsed with ice-cold phosphate-buffered saline and harvested in 800 μl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM $Na_2P_2O_7$, 5 mM EDTA) supplemented with protease inhibitors (9 nM pepstatin, 9 nM antipain, 10 nM leupeptin, and 10 nM

[2] In the present work, we use the terms NHERF1 (EBP50) and NHERF2 (E3KARP) to distinguish between the two forms of NHERF.

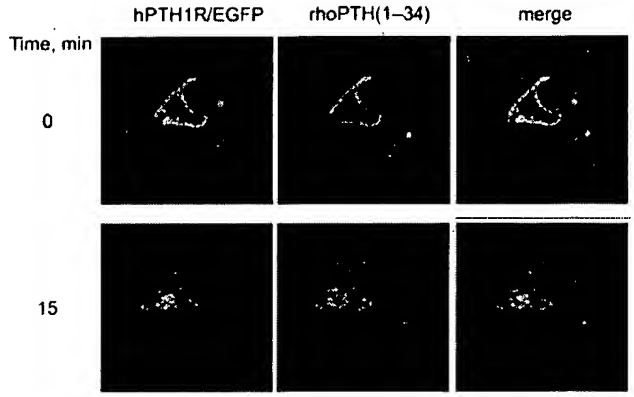

FIG. 1. **Colocalization of hPTH1R/EGFP and PTH-(1–34).** A single DCT cell stably expressing hPTH1R/EGFP is shown immediately upon (0 min) and 15 min after the addition of 10^{-7} M Rho-PTH-(1–34). At time 0, hPTH1R/EGFP (*green*) was largely membrane-delimited, although some trans-Golgi and perinuclear localization is visible. Rho-PTH-(1–34) (*red*) bound to PTH1R/EGFP on the cell membrane. Merge of hPTH1R/EGFP and Rho-PTH-(1–34) depicts regions where the receptor and ligand colocalize (*yellow*). After 15 min of treatment with Rho-PTH-(1–34), plasma membrane fluorescence was reduced, whereas fluorescence of both the PTH1R/EGFP and Rho-PTH-(1–34) increased and colocalized within the cytoplasm.

chymostatin) (Sigma). After lysis for 60 min at 4 °C, the lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. Four μg of specific anti-GFP polyclonal antibody (Molecular Probes, Inc., Eugene, OR) were added to the supernatant. After a 60-min incubation at 4 °C, 50 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with 1 ml of lysis buffer. Immunoprecipitated proteins were eluted by the addition of 50 μl of SDS sample buffer followed by a 30-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting using specific antibodies.

Immunoblot Analysis—Cells were grown to confluence in T-25 flasks, trypsinized, and collected by centrifugation. The resultant cell pellet was resuspended in 500 μl of Nonidet P-40 lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). Total protein concentrations were measured (Bio-Rad Dc Protein Assay). 30 μg of lysate (solubilized in Laemmli sample buffer) were resolved on 10% polyacrylamide gels by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corp.) according to standard methods. Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in Tris-buffered saline plus Tween-20 (TBST), incubated with polyclonal anti-EBP50 antibody (Affinity Bioreagents) at 1:1000 dilution for 4 h at room temperature, washed, and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) at 1:5000 dilution for 1 h at room temperature. Protein bands were visualized with a luminol-based enhanced chemiluminescence substrate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistics—Data are presented as means ± S.E., where n indicates the number of independent experiments. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as the mean ± S.E. of n independent experiments. Paired results were compared by analysis of variance with post-test repeated measures analyzed by the Bonferroni procedure. Single comparisons to control were analyzed by Dunnett's test (Prism; GraphPad). Differences greater than $p \leq 0.05$ were assumed to be significant.

RESULTS

PTH1R and PTH Endocytosis—The PTH1R and PTH-(1–34) were simultaneously localized in DCT cells stably expressing a human PTH1R COOH-terminally tagged with the enhanced green fluorescent protein (PTH1R/C-EGFP) as previously described (17, 28, 30). Cells were exposed to rhodamine-tagged PTH-(1–34) (*rhoPTH(1–34)* in Fig. 1). Initially, the PTH1R was largely limited to the plasma membrane, although some cytoplasmic and perinuclear fluorescence is evident (Fig. 1). The ligand was restricted to the plasma membrane. When the fluorescence images for receptor (*green*) and ligand (*red*) were merged, only PTH1R at the plasma membrane was associated with ligand (*yellow*). After 15 min, little PTH1R or PTH-(1–34) remained at the plasma membrane. The decreases of PTH1R and PTH fluorescence at the plasma membrane were accompanied by concomitant increases of cytoplasmic PTH1R/C-EGFP and rhodamine-labeled PTH-(1–34) fluorescence. These results are consistent with the view that PTH and the PTH1R colocalize and internalize together in response to receptor occupancy.

Cell- and Ligand-specific Internalization of the PTH1R—We determined the kinetics of PTH1R internalization by real time, quantitative confocal fluorescence microscopy monitoring of membrane-delimited fluorescence intensity of the PTH1R/C-EGFP fusion protein in live cells. Upon the addition of 10^{-7} M PTH-(1–34), PTH1R internalization began after a latency of 6–7 min and reached 50% at 15 min (Fig. 2*A*). The results obtained on single cells by fluorescent imaging were independently corroborated by measuring internalization of radiolabeled PTH-(1–34) in large (>100,000) populations of cells. Ligand internalization paralleled that of the receptor. During the first 5 min, less than 5% of [^{125}I]PTH-(1–34) was sequestered and 50% was endocytosed by 15 min (Fig. 2*B*). These findings demonstrate that the PTH1R and PTH-(1–34) traffic together and are internalized in a spatially and temporally congruent manner. The results qualitatively and quantitatively validate the optical determination of PTH1R endocytosis.

PTH-(1–34) also internalized the PTH1R in kidney proximal tubule (PCT) cells (Fig. 2*A*). Internalization began promptly without delay in PCT cells but by 15 min reached levels equivalent (50%) to that in DCT cells.

We next examined the effects of the PTH antagonist PTH-(7–34) on PTH1R internalization. As expected, PTH-(7–34) (10^{-7} M) did not promote PTH1R internalization in PCT cells (Fig. 2*C*). However, PTH-(7–34) promptly and efficiently induced receptor sequestration in kidney DCT cells. PTH1R endocytosis evoked by PTH-(7–34) was greater (82 ± 4.2 *versus* 49 ± 3.7%, $p < 0.01$) and more rapid (t = 2.5 *versus* >8 min) than that elicited by PTH-(1–34). From concentration dependence curves, half-maximal internalization (EC_{50}) of the PTH1R occurred at 0.90×10^{-9} M PTH-(1–34) and 10^{-8} M for PTH-(7–34). We confirmed that PTH-(7–34) lacks agonist activity (31, 32). In DCT cells, 10^{-7} M PTH-(7–34) had no effect on either adenylyl cyclase or phospholipase C, whereas PTH-(1–34) activated both adenylyl cyclase and phospholipase C (21.4 ± 0.1- and 3.2 ± 0.5-fold, respectively). These results additionally substantiate that EGFP ligation to the intracellular tail of the PTH1R does not interfere with its signaling in DCT cells, as in HEK-293 cells or COS cells (17).

To verify that the observed ligand-specific internalization effects of PTH-(7–34) were not due to the presence of EGFP on the intracellular tail of the PTH1R/C-EGFP, identical experiments were performed with a PTH1R, where EGFP is located in the extracellular domain (PTH1R/N-EGFP) (26). We previously established that this receptor construct signals and traffics normally in response to PTH-(1–34) in LLC-PK_1 kidney cells (26). PTH1R/N-EGFP internalization induced by PTH-(7–34) was 76 ± 8.7% at 15 min and did not differ from that of the PTH1R/C-EGFP, 81 ± 4.2%. PTH-(1–34) also efficiently internalized the PTH1R/N-EGFP. Thus, the functional properties of the PTH1R/C-EGFP and PTH1R/N-EGFP are not different from the native receptor.

The actions of PTH-(1–84) and PTH-(7–84) were assessed to determine if the full-length circulating forms of PTH exerted similar effects on PTH1R internalization. At comparable peptide concentrations (10^{-7} M), these naturally occurring forms of PTH evoked PTH1R endocytosis in DCT cells that was indis-

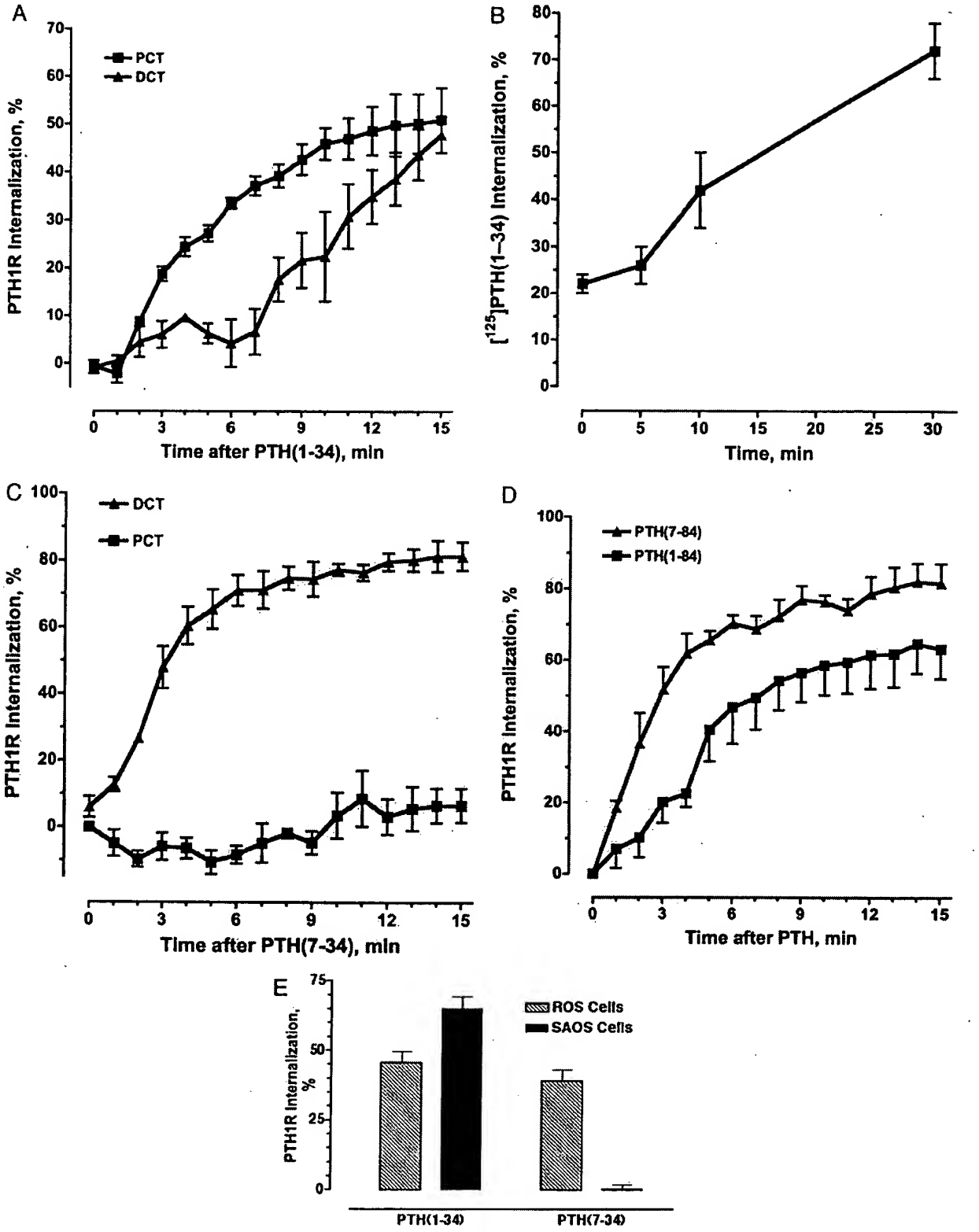

FIG. 2. **Ligand-induced PTH1R endocytosis.** *A*, effect of 10^{-7} M PTH-(1–34) on PTH1R internalization in PCT and DCT convoluted tubule cells. Receptor endocytosis was measured by real time quantitative confocal microscopy. Confocal images were quantified at 60-s intervals as described under "Experimental Procedures." $n = 3$ for PCT; $n = 4$ for DCT. *B*, radioligand internalization of PTH-(1–34) in DCT cells. Results are the means ± S.D. of triplicate determinations in two independent experiments. *C*, cell-specific PTH1R internalization by PTH-(7–34) in DCT but not PCT cells. 10^{-7} M PTH-(7–34) was added at 0 min. $n = 3$ for DCT; $n = 3$ for PCT. *D*, effect of full-length PTH peptides (10^{-7} M) on PTH1R endocytosis in DCT cells. *E*, ligand-induced PTH1R endocytosis in bone cells. PTH-(7–34) evokes PTH1R endocytosis in ROS cells but not in SaOS2 cells. Receptor endocytosis was measured after the addition of 10^{-7} M PTH-(1–34) or PTH-(7–34) as described. The extent of receptor endocytosis after 15 min is depicted. $n = 3$ for ROS; $n = 3$ for SaOS2.

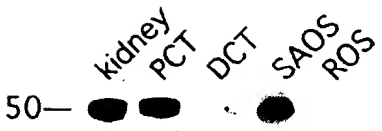

FIG. 3. **NHERF1 expression.** Immunoblot of mouse kidney, PCT, DCT, human osteosarcoma SaOS2, and rat osteosarcoma ROS 17/2.8 cells. Lanes were loaded with 5 μg (kidney) or 20 μg (cell lines) of protein per lane.

tinguishable from that displayed by their respective shorter synthetic analogs (Fig. 2*D*).

Similar cell-specific effects of PTH-(1–34) and PTH-(7–34) on PTH1R internalization were observed in two bone-derived cell lines, human SaOS2 and rat ROS 17/2.8 cells (Fig. 2*E*). In SaOS2 cells, as in PCT kidney cells, only PTH-(1–34) induced PTH1R internalization. In contrast, in ROS cells both PTH-(1–34) and PTH-(7–34) induced PTH1R endocytosis, similar to kidney DCT cells.

NHERF1 Expression Determines PTH-(7–34) Effects on PTH1R Endocytosis—NHERF1 is expressed by PCT and SaOS2 cells (Fig. 3), where PTH-(7–34) has no effect on PTH1R internalization, but not by DCT or ROS cells (Fig. 3), where PTH-(7–34) induces endocytosis (33, 34). Therefore, we theorized that the presence or absence of NHERF1 determines the cell-specific pattern of internalization of PTH1R in response to inactive PTH peptides.

To test this idea, we introduced NHERF1 in cells normally lacking it and determined the effect of PTH-(7–34) on PTH1R internalization. DCT and ROS cells were stably transfected with NHERF1. DCT/NHERF1 cells exhibited PTH1R internalization in response to PTH-(1–34) similar to that seen in vector-transfected or non-transfected control cells (Fig. 4*A*). Now, however, PTH1R internalization in response to PTH-(7–34) was significantly attenuated (Fig. 4*A*). PTH-(7–34)-induced PTH1R internalization was also largely inhibited in ROS cells stably expressing NHERF1 (Fig. 4*B*). PTH-(1–34) and PTH-(7–34) promoted the internalization of a PTH1R tagged with EGFP in the extracellular domain (PTH1R/N-EGFP) (Fig. 4*C*). NHERF1 similarly inhibited PTH-(7–34)-induced internalization of the PTH1R/N-EGFP as it did the PTH1R/C-EGFP (Fig. 4*C*). PTH-(1–34)-stimulated PTH1R/N-EGFP endocytosis was not affected. Thus, the actions of NHERF1 are independent of the location of EGFP within the PTH1R.

The role of NHERF1 in determining sensitivity to PTH-(7–34) was further and independently established by expressing a dominant negative form of NHERF1 (NHERH-(1–326), NHERF1ΔERM) (35, 36) in PCT cells that endogenously express NHERF1. Cells transfected with NHERF1ΔERM now displayed PTH1R internalization in response to PTH-(7–34) (Fig. 5), whereas they are normally refractory to PTH-(7–34), as shown by the cells transfected with empty vector.

Direct Interaction between NHERF1 and PTH1R Determines PTH-(7–34) Effects on PTH1R Endocytosis—The interaction between PTH1R and NHERF1 was directly demonstrated in HEK-293 cells co-expressing EGFP-tagged wild-type PTH1R with HA-tagged NHERF1. HEK-293 cells were used because they are normally devoid of PTH1R and have been previously employed for GPCR coimmunoprecipitation with NHERF1 (37, 38). Cell lysates were immunoprecipitated with a GFP-specific polyclonal antibody, and blotting was performed with an HA-specific monoclonal antibody. As shown in Fig. 6 (*top*), NHERF1 efficiently coimmunoprecipitated with the PTH1R. This finding also establishes that such interaction occurs constitutively (*i.e.* without ligand occupancy).

We then generated a full-length PTH1R/EGFP construct wherein the terminal methionine of the PTH1R was changed to

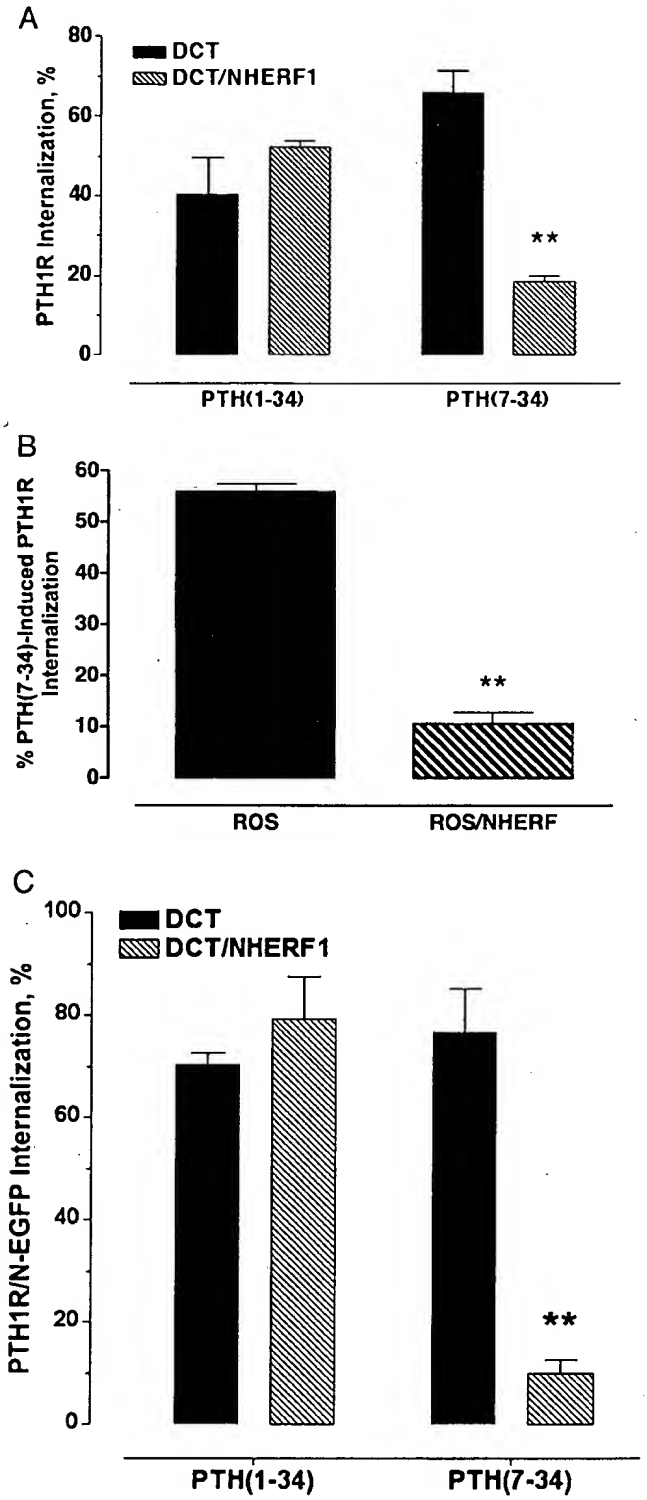

FIG. 4. **NHERF expression inhibits PTH1R endocytosis in DCT and ROS cells.** *A*, effect of 10^{-7} M PTH-(1–34) or PTH-(7–34) on PTH1R internalization in DCT cells after 15 min in the absence (*filled bars*) or presence (*stippled bars*) of NHERF1 (n = 3). **, $p < 0.01$ *versus* DCT. *B*, effect of 10^{-7} M PTH-(7–34) on PTH1R internalization in ROS cells in the absence (*filled bars*) or presence (*stippled bars*) of NHERF1. Receptor endocytosis was measured and quantified as outlined in Fig. 1*A*. The results show the average ± S.E. of three independent observations for each condition. **, $p < 0.01$ *versus* ROS. *C*, PTH-(7–34)-induced internalization of the PTH1R with an extracellular EGFP tag (PTH1R/N-EGFP). n = 3. **, $p < 0.01$ *versus* PTH-(1–34).

alanine (M593A) (ETVA-PTH1R/C-EGFP), a modification that disrupts the interaction of the PTH1R with the PDZ domain of NHERF1 (23). The ETVA-PTH1R/C-EGFP was expressed in a functional form and stimulated cAMP production in response to 10^{-7} M PTH-(1–34) similarly to wild type PTH1R (7.5 ± 0.5- and 8.5 ± 0.7-fold above basal level for ETVA-PTH1R and wild-type PTH1R, respectively).

In contrast to the wild-type ETVM-PTH1R, however, ETVA-PTH1R coimmunoprecipitation with NHERF1 was negligible (Fig. 6, *top*). Immunoblotting of the cell lysates with an anti-GFP antibody showed that both wild-type and ETVA-PTH1R were expressed at similar levels (Fig. 6, *middle*). Likewise, immunoblotting of the cell lysates with the HA-specific antibody showed that HA-NHERF1 expression levels were similar in all conditions (Fig. 6, *bottom*). Thus, mutation of a single residue of the PDZ recognition domain was sufficient to disrupt the association of the PTH1R with NHERF1. The results further demonstrate that the presence of EGFP at the carboxyl terminus of the PTH1R does not occlude or interfere with the PDZ-recognition domain, binding to NHERF1, or PTH1R signaling.

The ETVA-PTH1R was used to test the hypothesis that disrupting the PDZ recognition domain of the PTH1R, which prevents binding to NHERF1, would permit internalization in response to PTH-(7–34). This was accomplished by introducing the ETVA-PTH1R/C-EGFP in PCT cells that constitutively express NHERF1. Fig. 7*A* shows that 10^{-7} M PTH-(7–34), which normally has a negligible effect on PTH1R internalization, now effectively internalized the ETVA-PTH1R. PTH-(1–34) stimulated internalization of the ETVA-PTH1R as efficiently as that of the wild-type PTH1R. Comparable results were obtained with ROS/NHERF cells (Fig. 7*B*). These experiments provide strong evidence that the PDZ-binding domain of the PTH1R is necessary and sufficient to mediate the association with NHERF1. Moreover, they establish that disrupting this interaction confers sensitivity to PTH-(7–34).

Role of ERM Domain on PTH1R Endocytosis—NHERF1 possesses a carboxyl-terminal ERM domain. To determine whether the ERM domain of NHERF1 is required for ligand-induced PTH1R internalization, we expressed ERM-deficient NHERF1 (NHERFΔERM) in DCT cells. In contrast to full-length NHERF1, which substantially inhibited PTH1R internalization initiated by PTH-(7–34), NHERFΔERM exerted no significant inhibitory action (Fig. 8). This finding is consistent with the idea that NHERF1 tethers the PTH1R to the actin cytoskeleton through the ERM domain of NHERF1 and, in the absence of the ERM domain, the PTH1R is unconstrained. In this setting, receptor occupancy by PTH is sufficient to induce endocytosis.

The ERM domain of NHERF1 binds actin-associated proteins (39). We reasoned that if the PTH1R is tethered through NHERF to ezrin and the actin cytoskeleton, disrupting the actin cytoskeleton should unleash the PTH1R from NHERF1 and permit PTH-(7–34) to internalize the receptor. We tested this theory in PCT cells because they express NHERF1 and because PTH-(7–34) normally has a minimal effect on PTH1R endocytosis. Treatment with 1 μM cytochalasin D, a membrane-permeant inhibitor of actin polymerization, fully allowed PTH-(7–34) to promote receptor internalization (Fig. 9). Since the ETVA-PTH1R does not interact with NHERF1, cytochalasin D should not interfere with PTH-(7–34)-initiated PTH1R sequestration. As predicted, actin depolymerization did not alter PTH-(7–34)-stimulated internalization of the ETVA-PTH1R (Fig. 9). In contrast to the inhibitory action of cytochalasin D on PTH (7–34)-induced PTH1R internalization, microtubule disruption with colchicine (1 μM) had no effect on PTH1R inter-

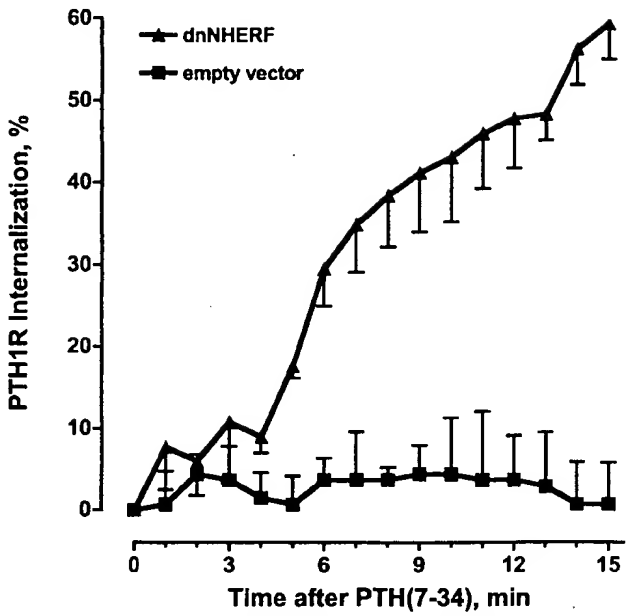

FIG. 5. **ERM-deficient dominant negative NHERF (NHERF-(1–326)) permits PTH-(7–34) to internalize the PTH1R in PCT cells.** The effect of 10^{-7} M PTH-(7–34) on PTH1R internalization in PCT cells in the presence or absence of NHERF-(1–326). Receptor endocytosis was measured and quantified as outlined in Fig. 1*A*. $n = 4$ for each condition.

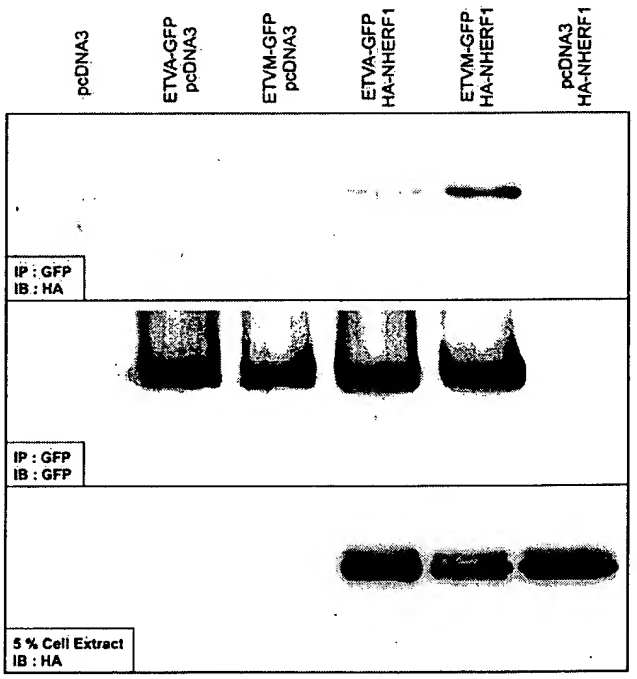

FIG. 6. **Coimmunoprecipitation of EBP50 with PTH1R(ETVM)-EGFP and PTH1R(ETVA)-EGFP.** HEK-293 cells transfected with the indicated constructs were harvested, lysed, and immunoprecipitated as described under "Experimental Procedures." Immunoprecipitation experiments were performed by incubating the cell lysates with an EGFP-specific polyclonal antibody followed by incubation with protein A-agarose. Immunoprecipitated proteins were eluted from protein A-agarose with SDS sample buffer. Eluates and cell extracts were subjected to SDS-PAGE. Immunoblotting was performed with a HA-specific monoclonal antibody (*upper panel*). The amount of immunoprecipitated EGFP-tagged receptor in each sample was verified by immunoblotting with the EGFP-specific polyclonal antibody (*middle panel*). The quantity of HA-EBP50 present in each cell extract was evaluated by immunoblotting with the HA-specific monoclonal antibody (*lower panel*). A representative Western blot is shown.

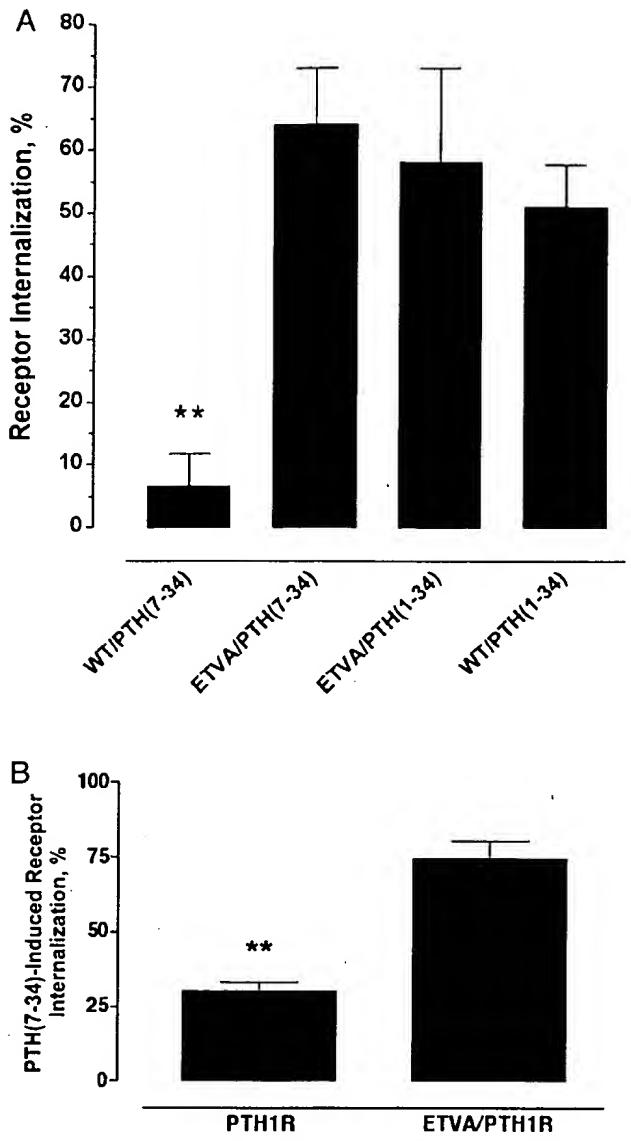

FIG. 7. **Mutation of the carboxyl-terminal PDZ interaction domain in the PTH1R abolishes NHERF-mediated inhibition of PTH-(7–34)-induced PTH1R endocytosis.** *A*, PCT cells expressing either the wild-type (*WT*) PTH1R/EGFP or mutated (*ETVA*) PTH1R/EGFP were challenged with 10^{-7} M PTH-(1–34) or PTH-(7–34) for 15 min as indicated. n = 3–4 for each condition. **, $p < 0.01$ *versus* wild-type PTH-(1–34). *B*, ROS/NHERF cells expressing either the wild-type PTH1R/EGFP (n = 4) or mutated ETVA-PTH1R/EGFP (n = 3) were challenged with 10^{-7} M PTH-(7–34), and endocytosis was measured as described. **, $p < 0.01$ *versus* ETVA-PTH1R.

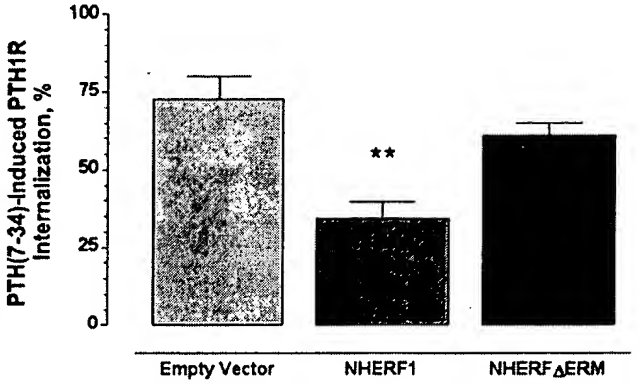

FIG. 8. **ERM-deficient NHERF-(1–326) does not inhibit PTH-(7–34)-induced PTH1R endocytosis in DCT cells.** DCT cells were transiently transfected with PTH1R/EGFP in the presence of pcDNA 3.1 (empty vector), NHERF1, or ERM-deficient NHERF-(1–326), and PTH1R internalization was assessed after challenge with 10^{-7} M PTH-(7–34) for 15 min. n = 3 for each condition. **, $p < 0.01$ *versus* empty vector.

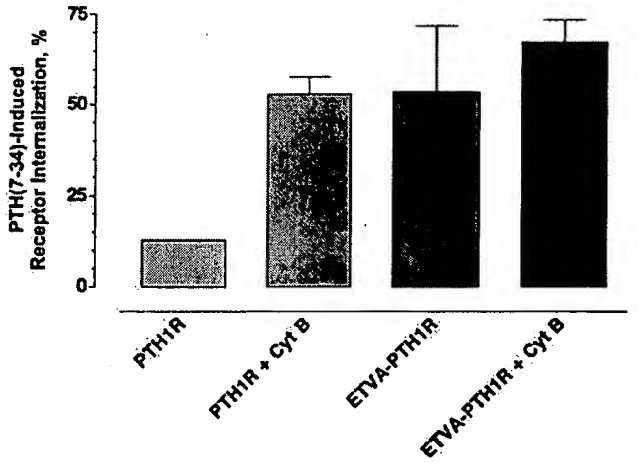

FIG. 9. **Disruption of the actin cytoskeleton permits PTH-(7–34)-induced PTH1R internalization in PCT cells.** PCT cells were transiently transfected with PTH1R/EGFP or ETVA-PTH1R/EGFP. Where indicated, cells were treated with 1 μM cytochalasin D for 15 min before the addition of 10^{-7} M PTH-(7–34). Receptor endocytosis was then measured after 15 min and quantified as described. Results show internalization at t = 15 min.

nalization (data not shown). These results support the view that the presence of an intact actin network and NHERF1 determine the fate of PTH1R trafficking in response to activating or inactivating PTH peptides.

PTH-(7–34)-induced PTH1R Internalization Is Independent of β-Arrestin but Requires Dynamin—To define further the molecular mechanisms underlying antagonist-induced PTH1R endocytosis in DCT cells, we sought to determine whether PTH1R sequestration involves β-arrestin and dynamin. DCT cells were transiently transfected with β-arrestin-2-GFP and monitored by fluorescence microscopy. Under resting conditions, β-arrestin-2 was uniformly distributed throughout the cytoplasm but excluded from the nucleus (Fig. 10, *top*). Within 5 min of the addition of 10^{-7} M PTH-(1–34), β-arrestin-2 moved from the cytoplasm to the plasma membrane, exhibiting a characteristic punctate distribution. By 25 min, β-arrestin had translocated to the cytoplasm. This observation is consistent with arrestin-mediated trafficking of the PTH1R to clathrin-coated pits for internalization. In contrast, PTH-(7–34) (10^{-7} M) exerted no detectable effect on β-arrestin-2 movement (Fig. 10, *bottom*).

DCT cells expressing PTH1R-EGFP were transfected with a dominant-negative form of dynamin, [K44A]dynamin (40). In these cells, PTH1R internalization was significantly inhibited, both in response to the agonist PTH-(1–34) and to the antagonist PTH-(7–34) (Fig. 11). Hence, whereas agonist-occupied PTH1R internalizes in a classical β-arrestin- and dynamin-dependent fashion, receptor occupancy by the nonactivating analog PTH-(7–34) induces PTH1R endocytosis independently of β-arrestin. Dynamin function, however, is required.

DISCUSSION

A feature common to GPCRs is the cyclical process of activation, desensitization and internalization, resensitization, and recycling to the plasma membrane (41). These coordinated events protect against excessive receptor stimulation or periods of prolonged inactivity. In this manner, receptor activation,

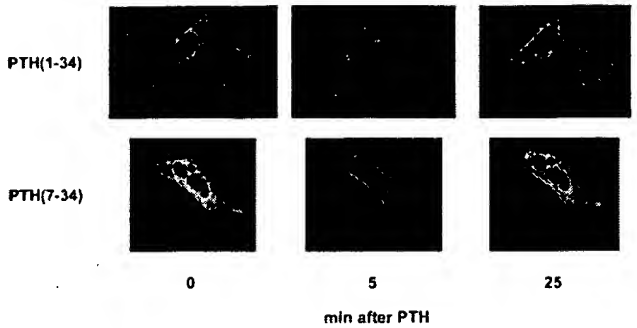

FIG. 10. **PTH-(1–34) translocates β-arrestin-2 in DCT cells.** DCT cells were transiently transfected with β-arrestin-2-GFP and hPTH1R as outlined under "Experimental Procedures." Real time, live cell confocal images were taken after 0, 5, and 25 min of treatment with 10^{-7} M PTH-(1–34) or -(7–34), as indicated.

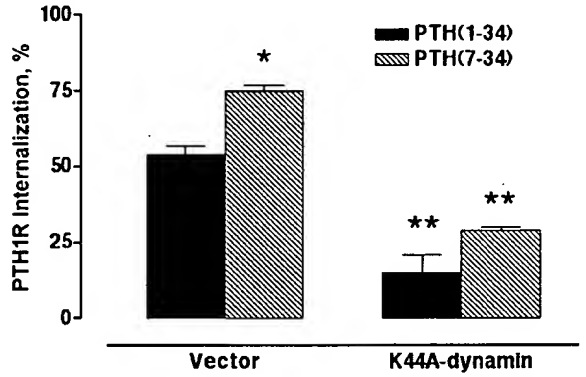

FIG. 11. **PTH1R endocytosis is dynamin-dependent.** DCT cells were transiently transfected with PTH1R/EGFP in the presence of dominant negative K44A-dynamin or empty vector. PTH1R internalization was analyzed after treatment for 15 min with 10^{-7} M PTH-(1–34) or PTH-(7–34) as indicated. $n = 3$ independent observations for each condition. *, $p < 0.05$; **, $p < 0.01$ *versus* vector plus PTH-(1–34).

desensitization, and trafficking are thought to go hand-in-hand, thereby regulating the physiological balance of GPCR activity. In some instances, however, GPCR activation can be dissociated from subsequent desensitization and internalization. For the PTH1R, this has been shown with synthetic analogs of the PTH-related protein that stabilized an active, G protein-coupled PTH1R state. In this configuration, persistent signaling is maintained, and the receptor is not competent to interact with β-arrestin-2 and is not desensitized or internalized (17). Conversely, PTH1R mutants have been generated that exhibit severely blunted signaling activities but are phosphorylated by G protein-coupled receptor kinase 2 and endocytosed in a β-arrestin-2-dependent manner (18). Similar findings were reported following mutagenesis of β_2-adrenergic (42) and angiotensin type 1 receptors (43).

This raises the important question of whether ligands that bind but do not activate signaling through G proteins are capable of inducing receptor sequestration. In the present studies, this possibility was explored by using structural analogs of PTH on cells derived from kidney (proximal and distal tubule cells) and bone (SaOS2 and ROS cells), since these organs are major targets of PTH action. We found that PTH1R internalization occurs in response to NH_2-terminally truncated fragments of PTH that do not activate signaling either through G_s or G_q proteins. Strikingly, this PTH1R internalization occurs in a markedly cell-specific fashion and proceeds through a mechanism that is distinct from that induced by PTH agonists. The agonist PTH-(1–34) activated the PTH1R, mobilized β-arrestin, and internalized the receptor, as previously shown in HEK-293, COS-7, and LLC-PK_1 cells (11, 44, 45). PTH-(7–34), a competitive inhibitor of the PTH1R, however, robustly promoted receptor internalization in DCT and ROS cells without accompanying activation or β-arrestin-2 translocation. These results demonstrate that PTH1R activation and internalization can be dissociated in a ligand- and cell-selective fashion. Some precedent for this phenomenon may be found with the class A serotonin, endothelin, and cholecystokinin GPCRs, where both agonists and synthetic antagonists internalize these receptors (46–48). It has been suggested that the distinct biological effects of different ligands acting through a common GPCR partially depend upon their abilities to induce endocytosis (49). Thus, the dissociation between receptor activation and internalization as shown here may represent a more common biological phenomenon that contributes to ligand- and cell-specific hormone and drug action for multiple classes of GPCRs.

NHERF1 has been shown previously to affect the function of some GPCRs containing PDZ-binding motifs. NHERF1, for instance, enhances the rate of recycling of K-opioid (36) and β_2-adrenergic receptors (50). Disrupting the interaction of NHERF1 with the β_2-adrenergic receptor causes sorting of endocytosed receptor to the lysosomal pathway instead of the recycling pathway (50). Segre and co-workers (23) reported that NHERF1 and -2 bind to the PTH1R through a COOH-terminal PDZ-binding domain (ETVM) and determined a role for NHERF2 in PTH signaling. The present work illustrates a different role of NHERF1 that is distinct from its effect on receptor recycling, on the one hand, and apparently unrelated to the signaling switch, on the other.

The capacity of NHERF1 to establish cell-specific effects on PTH1R internalization was tested in three independent ways. In the first, we mutated the PDZ-binding domain of PTH1R by changing the terminal methionine to alanine (M593A). This mutation is sufficient to abolish the association of the full-length PTH1R with NHERF1 (Fig. 6). The ETVA-PTH1R was fully functional, and, as expected, in cells lacking NHERF1 was endocytosed equivalently to the wild-type PTH1R by both PTH-(1–34) and PTH-(7–34). The ETVA-PTH1R, however, was also efficiently internalized in response to PTH-(7–34) in cells expressing NHERF1, whereas the wild-type PTH1R was not. Thus, interfering with the ability of the PTH1R to associate with NHERF1 is sufficient to permit the nonsignaling PTH-(7–34) to internalize the receptor.

PTH1R fusion proteins containing EGFP within the extracellular domain or at the carboxyl-terminus of the receptor were used. These receptor constructs exhibit signaling behavior that is indistinguishable from the native receptor (26, 30). As shown here, the EGFP-tagged receptors were both internalized in response to PTH-(7–34), and this effect was absent in cells constitutively expressing or transfected with NHERF1. Further, the wild-type PTH1R sequence COOH-terminally ligated to EGFP efficiently coimmunoprecipitated with NHERF1. Single residue mutagenesis of the terminal Met of the PTH1R was sufficient to abrogate interaction of the PTH1R/C-EGFP with NHERF1. Therefore, the interaction of NHERF1 with the PTH1R/EGFP fusion protein involves the ETVM recognition motif within the PTH1R sequence. These findings further indicate that COOH-terminal ligation of EGFP does not interfere with the physical interaction or functional effects of NHERF1 with the PTH1R. In this regard, the EGFP-tagged PTH1R behaves like certain other proteins such as nNOS that recognize internal PDZ motif-mediated interactions (51). An 18-amino acid linker between the COOH terminus of the PTH1R sequence and the start of the EGFP sequence may

facilitate interaction between the PDZ recognition motif and NHERF1. The two PDZ domains of NHERF1 can dimerize, preferentially through homologous binding interactions (52, 53). NHERF1 dimerization may permit interaction with non-canonical COOH-terminal PDZ recognition motifs as in the PTH1R/EGFP fusion protein.

The second strategy to examine the role of NHERF1 in regulating PTH1R endocytosis involved using a truncated form of NHERF1 that lacks the ERM domain (NHERF-(1–326)) but contains both PDZ domains. In cells expressing NHERF1, NHERF-(1–326) exerted a dominant-negative function and permitted PTH-(7–34) to internalize the PTH1R. Furthermore, whereas introduction of full-length NHERF1 in cells normally lacking it suppressed the effect of PTH-(7–34) (Fig. 4, *A* and *B*), expression of NHERF-(1–326) alone had no effect (Fig. 8). These results further establish a role for the NHERF1 ERM domain in tethering the PTH1R and indicate that interfering with the association between PTH1R, NHERF1, and the actin cytoskeleton allows the occupied, but not activated, receptor to be endocytosed.

ERM proteins such as NHERF1 contain an F-actin binding site in the COOH-terminal 28 residues (39). Ezrin, a member of the ERM family, cross-links the actin cytoskeleton to the plasma membrane. Ezrin is abundantly expressed at the apical brush-border membrane of proximal tubules, the site of NHERF1 localization (34, 54, 55). Ezrin is likewise expressed by osteosarcoma cells (56). Therefore, the third tactic that was applied to test the role of NHERF1 in determining the effects of inactive ligands on PTH1R internalization was to disrupt the actin cytoskeleton. Application of cytochalasin D to cells possessing NHERF1 allowed PTH-(7–34) to internalize the PTH1R. This effect was quite specific for the actin cytoskeleton, since microtubule disruption with colchicine had no effect on PTH1R internalization. These independent approaches provide strong evidence that NHERF1 dictates the response of the PTH1R to occupancy by nonactivating PTH peptides. In cells lacking NHERF1, PTH1R occupancy is sufficient to promote receptor internalization without prior or concurrent activation.

The mechanism of PTH1R internalization in response to PTH-(7–34) in cells lacking NHERF1 is, at least in part, different from that commonly employed by the same receptor in response to agonists. Agonist-induced endocytosis of the PTH1R occurred in an arrestin-dependent manner. In the case of nonactivating analogs, PTH1R sequestration proceeds in a β-arrestin-independent manner. In both instances, however, internalization requires dynamin. This distinct internalization pathway is operative in cells lacking NHERF1 or in cells expressing NHERF1ΔERM. Taken together, these findings suggest that the role of NHERF1 is not necessarily an "active" one but rather that the interactions between the PTH1R, NHERF1, and cytoskeleton (through the ERM domain) confer sufficient membrane stability on the PTH1R to require full agonist occupancy for internalization. This possibility is supported by the observation that the interaction between PTH1R and NHERF1 is constitutive, since both proteins coimmunoprecipitated from preparations of nontreated cells.

It is now apparent that PTH1R activation, desensitization, and internalization can be dissociated with each event requiring distinct receptor conformational states. These specific states can be selectively stabilized by appropriate modifications of the ligand. PTH analogs containing specific modifications at the NH_2 terminus have been shown to efficiently activate the PTH1R, but they fail to induce arrestin-mediated internalization (17). The postactivation response of the PTH1R depends on specific interactions between the NH_2-terminal activation domain of the ligand and the third extracellular loop of the receptor (17). These interactions are distinct from those necessary for activation of G protein-mediated signal transduction. The present work shows that the presence of the adaptor protein NHERF1 and its interaction with the PTH1R legislates the cell-specific pattern of PTH1R internalization in response to otherwise inactive PTH fragments. Taken together, these observations indicate that PTH1R activation and desensitization/endocytosis are mediated through distinct structural states that derive from specific interactions between ligand and receptor. Thus, agonist- or antagonist-occupied receptor states induce distinct conformations or accessibility to intracellular domains. The differential or inducible involvement of these domains in coupling to G proteins may represent a molecular basis for ligand-selective responses not only for the PTH1R but also for other GPCRs. In the case of the PTH1R, these conformational states also depend on interactions between the PTH1R and NHERF1 at the cytoplasmic surface.

In addition to its relevance to GPCR regulation and trafficking, the present studies may have implications for understanding extracellular calcium homeostasis. After synthesis of mature PTH-(1–84), the protein is concentrated in secretory vesicles and granules. Morphologically distinct granule subtypes contain both PTH and the proteases cathepsin B and cathepsin H (59). The co-localization of proteases and PTH in secretory granules may explain the observation that a portion of the PTH secreted from parathyroid glands consists of amino-terminally truncated PTH fragments (60). These fragments do not activate the PTH1R. Therefore, the intracellular fragmentation of PTH is thought to represent an inactivating pathway to dispose of "excess" peptide in situations such as hypercalcemia (61, 62). As shown here (Fig. 2*D*), PTH-(1–84) and PTH-(7–84) exerted actions on PTH1R internalization comparable with their shorter respective synthetic analogs. This novel finding suggests that PTH-(7–84) is not so much an inactive peptide as an inactivating protein.

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Human PTH-(7-84) Inhibits Bone Resorption *in Vitro* Via Actions Independent of the Type 1 PTH/PTHrP Receptor

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The linear sequence of intact mammalian PTH consists of 84 amino acids, of which only the most amino(N)-terminal portion, *i.e.* PTH-(1-34), is required for the classical actions of the hormone on mineral ion homeostasis mediated by the type 1 PTH/PTHrP receptor (PTH1R). Like the N-terminus, the carboxyl (C)-terminal sequence of PTH is highly conserved among species, and various circulating PTH C-fragments are generated by peripheral metabolism of intact PTH or are directly secreted, in a calcium-dependent manner, by the parathyroid glands. Certain synthetic PTH C-fragments exert actions on bone and cartilage cells that are not shared by PTH-(1-34), and specific binding of PTH C-peptides has been demonstrated in bone cells in which PTH1R expression was eliminated by gene targeting. The peptide human (h) PTH-(7-84) recently was shown to inhibit the calcemic actions of hPTH-(1-34) or hPTH-(1-84) in parathyroidectomized animals. To determine whether this anticalcemic effect of hPTH-(7-84) *in vivo* might result from direct actions on bone, we studied its effects on both resorption of intact bone *in vitro* and formation of osteoclasts in primary cultures of murine bone marrow. Human (h) PTH-(7-84) (300 nM) reduced basal 72-h release of preincorporated ^{45}Ca from neonatal mouse calvariae by 50% (9.6 ± 1.9% *vs.* 17.8 ± 5.7%; $P < 0.001$) and similarly inhibited resorption induced by hPTH-(1-84), hPTH-(1-34), 1,25-dihydroxyvitamin D_3 (VitD), PGE_2, or IL-11. In 12-d murine marrow cultures, both hPTH-(7-84) (300 nM) and hPTH-(39-84) (3000 nM) lowered VitD-dependent formation of osteoclast-like cells by 70%. On the contrary, these actions of hPTH-(7-84) were not observed with the PTH1R antagonists hPTH-(3-34)NH_2 and [L^{11},D-W^{12},W^{23},Y^{36}]hPTHrP-(7-36)NH_2, which, unlike hPTH-(7-84), did inhibit PTH1R-dependent cAMP accumulation in ROS 17/2.8 cells. We conclude that hPTH-(7-84), acting via receptors distinct from the PTH1R and presumably specific for PTH C-fragments, exerts a direct antiresorptive effect on bone that may be partly due to impaired osteoclast differentiation. (*Endocrinology* 143: 171–176, 2002)

INTACT PTH from different mammalian species comprises 84 amino acids, the sequence of which is highly conserved within both its amino (N)-terminal and carboxyl (C)-terminal regions (1). The first 34 amino acids of PTH, including an intact N-terminus, are both necessary and sufficient for the classical actions of the hormone on mineral ion homeostasis and bone metabolism. These effects of intact and N-terminal PTH are mediated through the type 1 PTH/PTH-related peptide receptor (PTH1R), a G protein-coupled receptor that can activate both adenylate cyclase and PLC (2).

The parathyroid glands are the main source of PTH, although small amounts of its mRNA were recently identified in hypothalamus and spleen (3). PTH synthesis and secretion are tightly controlled by calcium via a membrane-bound calcium-sensing receptor (4), although vitamin D (5, 6) and phosphate (7, 8) also play modulating roles. Under physiological conditions, a portion of the newly synthesized hormone undergoes intraglandular cleavage at a rate that also is regulated by extracellular calcium (9, 10). This cleavage results in the cosecretion of intact PTH and various C-terminal fragments, the predominant forms of which, identified to date, consist of peptides with N-termini located between residues 24 and 43 (11–13). Secreted intact PTH also undergoes endopeptidic cleavage(s) in peripheral tissues, mainly liver and kidney, by processes that degrade the resulting N-terminal fragments *in situ* but release additional C-fragments into the circulation (14–16). As a consequence of their obligatory renal clearance, the concentration of circulating C-terminal PTH (CPTH) fragments increases dramatically in patients with renal failure (17–19).

Recently, fragments of PTH lacking residues at the extreme N-terminus but otherwise large enough to cross-react with most commercially available intact PTH two-site immunoassays were detected after HPLC fractionation of normal plasma and, at much higher levels, in plasma of patients with advanced renal failure (19). Although their precise structure(s) has not been ascertained, these fragments exhibit chromatographic properties similar to those of synthetic PTH-(7-84) (18). Interestingly, human (h) PTH-(7-84) was recently shown to inhibit the calcemic actions of PTH-(1-84) and PTH-(1-34) in parathyroidectomized animals at doses much lower than would be predicted to effectively antagonize either hormonal form at the PTH1R (20, 21). Thus, these *in vivo* observations suggest that CPTH fragments might act upon bone cells via one or more mechanisms independent of the PTH1R *per se*.

The possibility that CPTH fragments (as well as intact PTH) might activate receptors distinct from the PTH1R was first postulated over 2 decades ago when Arber *et al.* (22) showed that a particular CPTH fragment, PTH-(53-84), possessed biological properties different from those of PTH-(1-34). Subsequent work from several different groups has produced direct evidence that CPTH fragments from within the sequence PTH-(35-84) bind specifically to bone and kidney cells and/or membranes and can exert direct actions on target cells in bone or cartilage. For example, CPTH frag-

Abbreviations: CPTH, C-terminal PTH; hPTH, human PTH; PTH1R, type 1 PTH/PTHrP receptor; TRAP, tartrate-resistant acid phosphatase; TRAP+MNC, TRAP-positive cells containing three or more nuclei (osteoclast-like multinucleated cells); VitD, 1,25-dihydroxyvitamin D_3.

ments such as hPTH-(53-84) and hPTH-(60-84) increased alkaline phosphatase activity and expression of mRNAs for both alkaline phosphatase and osteocalcin in bone-derived cells and induced transient increases in cytosolic free calcium in chondrocytes (23–26). Photoaffinity cross-linking studies to characterize the receptors for CPTH fragments (*i.e.* CPTHRs) expressed by ROS 17/2.8 osteosarcoma and rPT parathyroid cells were performed by Inomata *et al.* (27) using radioiodinated ($Leu^{8,18}$,Tyr^{34})hPTH-(1-84) and (Tyr^{34})hPTH-(19-84), neither of which binds well, if at all, to the PTH1R. These studies showed that in ROS 17/2.8 cells, two proteins (80 and 30 kDa) interacted specifically with the radioligands used, whereas in rPT cells, only the 80-kDa protein was observed. Affinity labeling was inhibited by hPTH-(1-84), hPTH-(19-84), and, to a lesser extent, by CPTH fragments that were truncated even further at the N-terminus, whereas hPTH-(1-34) had no effect (27). Recently, hPTH-(7-84) was shown to bind to CPTHRs on ROS 17/2.8 cells with affinity comparable to that of hPTH-(1-84) (21).

Unequivocal evidence that such CPTHRs are distinct from the PTH1R was provided by our recent demonstration that specific [^{125}I](Tyr^{34})hPTH-(19-84) binding is observed in clonal osteoblastic and osteocytic cell lines derived from mice in which the PTH1R gene had been eliminated by gene targeting (28). Further, CPTH fragments such as hPTH-(39-84) were shown to regulate cellular functions (*i.e.* connexin 43 expression and apoptosis) in clonal PTH1R-null osteocytes at concentrations shown to bind effectively to CPTHRs in these cells (28).

Thus, the expression of CPTHRs in bone offers a plausible mechanism by which circulating PTH fragments, truncated at their N-termini and including peptides as long as hPTH-(7-84) might exert biological actions, potentially different from those of intact PTH, by a means other than direct antagonism at the PTH1R. To determine whether the ability of hPTH-(7-84) to antagonize the calcemic response to PTH-(1-84) *in vivo* might reflect direct actions of this C-PTH fragment on bone, we studied its effects using *in vitro* assays of osteoclast formation and bone resorption.

Materials and Methods

Materials

Culture media were obtained from the Media Kitchen (Pediatric Surgery, Massachusetts General Hospital, Boston, MA), other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), and additional reagents and chemicals were obtained from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA). Recombinant hPTH-(1-84) was a gift from Chugai Pharmaceutical Co. (Shizuoka, Japan), and hPTH-(7-84) and [D^{76}]hPTH-(39-84) were purchased from Bachem (Torrance, CA). All other PTH fragments, including the PTH1R antagonist (Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36})hPTHrP-(7-36)amide PTHrP-(7-36) (29, 30) and hPTH-(3-34)-amide, were synthesized at Massachusetts General Hospital Peptide and Oligonucleotide Core Laboratory (Boston, MA). Recombinant mouse IL-11 was purchased from R&D Systems, Inc. (Minneapolis, MN), and VitD was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Animals

Animals were maintained in facilities operated by the Massachusetts General Hospital Center for Comparative Resources in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institutional animal care and use committee.

Bone resorption assay

Bone resorption was quantitated by the release of previously incorporated ^{45}Ca from newborn mouse calvarial bones *in vitro* (31). Briefly, calvaria from 3- to 4-d-old mice (CD-1 strain, Charles River Laboratories, Inc., Wilmington, MA) were obtained after maternal administration of 50 μCi $^{45}CaCl_2$ (NEN Life Science Products, Boston, MA), sc, on the 19th day of gestation. The bones were divided in half and precultured in 1 ml DMEM containing 1 mM calcium, 2 mM phosphate, 5% heat-inactivated horse serum, and 1% antibiotic/antimycotic solution (Life Technologies, Inc.) on a rocking platform at 90 oscillations/min in a 37 C incubator under 5% CO_2 in air. After 24 h the medium was replaced with 1 ml fresh medium containing the test substances (or vehicle alone). After an additional 72 h, the bones were removed, rinsed three times in PBS, placed in scintillation vials containing 0.4 ml 2 N HCl, and incubated for 2 h at room temperature before addition of 5 ml scintillation fluid (Packard Instruments, Downers Grove, IL). Aliquots of culture medium (0.5 ml) were transferred to separate vials containing 5 ml scintillation fluid for determination of released radioactivity. In some experiments additional aliquots of culture medium were used for measurements of cAMP as described below. Bone resorption was determined as the percentage of total initial bone ^{45}Ca subsequently released into the medium during the 72-h treatment period. Results are expressed as the mean ± SEM of the percentage of ^{45}Ca released for groups of four bones and are representative of at least three independent experiments.

Bone marrow culture

Bone marrow cells were isolated as previously described (32). Briefly, 4- to 6-wk-old male mice (C57B/6 strain, Charles River Laboratories, Inc.) were killed by carbon dioxide asphyxiation, and tibias and femurs were aseptically removed and dissected free of adhering tissue. The metaphyses were removed, and the marrow cavity was flushed with 1 ml αMEM to obtain marrow cells, which were collected into 50-ml tubes and washed twice with αMEM. Cells were cultured in growth medium [αMEM containing 10% FBS (lot 1011961 Life Technologies, Inc.) and 1% penicillin-streptomycin] containing 100 nM dexamethasone (Sigma) after plating at 1.5×10^6 cells/well in 24-well plates. Half of the culture medium was replaced 3 times/wk with fresh medium containing a 2× concentration of the test substances (or vehicle). All cultures were maintained in a 37 C incubator under 5% CO_2 in air. After culture for 10 d, cells adherent to the surface of each well were rinsed twice with PBS, fixed with 10% formalin in PBS for 10 min at room temperature and with ethanol/acetone (50:50, vol/vol) for 1 min before staining for tartrate-resistant acid phosphatase (TRAP), as previously described (33). TRAP-positive cells containing 3 or more nuclei were scored as osteoclast-like multinucleated cells (TRAP+MNCs). Cells were counted at ×10 magnification in 20 contiguous fields along 2 orthogonal pathways in each well, a method previously employed to account for the nonuniform distribution of cells within wells (33). The number of TRAP+MNCs contained in these 20 fields was expressed as the number per well.

cAMP accumulation

Clonal rat osteosarcoma cell (ROS 17/2.8) were cultured in 48-well plates in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. The cultures were maintained for 5–7 d after reaching confluence by replacing the medium every other day. To assess basal and agonist-induced cAMP accumulation, cells were rinsed twice with assay buffer (DMEM containing 2 mM isobutylmethylxanthine, 1 mg/ml heat-inactivated BSA, and 35 mM HEPES-NaOH, pH 7.4) and then incubated for 45 min at 23 C with the same buffer alone or in the presence of different peptides (or with conditioned medium collected from resorption assays). The buffer then was rapidly aspirated, the plates were frozen on powdered dry ice, and the frozen cells were subsequently thawed directly into 0.25 ml 50 mM HCl. Cell-associated cAMP in the acid extracts was measured as previously described (34). Results were expressed as picomoles of cAMP produced per well over 45 min.

Statistical analysis

Results are expressed as the mean ± SEM or the mean ± SD. The significance of differences between treatment and control groups was

assessed by the Mann-Whitney test. Data were analyzed using the PRISM 3.0 software package for Macintosh (GraphPad Software, Inc., San Diego, CA).

Results

The limited efficacy of short, amino-terminally truncated PTH or PTHrP analogs, such as hPTH-(3-34) or hPTHrP-(7-36), to antagonize the action of PTH-(7-34) or PTH-(1-84) *in vivo* or *in vitro* contrasts with the efficiency with which hPTH-(7-84) inhibits the calcemic action of hPTH-(1-84) [or hPTH-(7-34)] *in vivo* (20, 21). Although pharmacokinetic differences *in vivo* between N-truncated PTH fragments of different length and structure might explain these differences, we considered the alternative possibility that, unlike short N-truncated PTH or PTHrP fragments, this effect of hPTH-(7-84) *in vivo* might not be mediated via antagonism at the PTH1R. We therefore directly compared the effects of hPTH-(7-84) with those of PTHrP-(7-36) or hPTH-(3-34) in an *in vitro* assay of bone resorption that relies upon the release of pre-incorporated ^{45}Ca from neonatal murine calvarial bones. First, as shown in Fig. 1, addition of hPTH-(7-84) (300 nM) alone reduced basal ^{45}Ca release by approximately 50% [control, 17.8 ± 5.7%; hPTH-(7-84), 9.6 ± 1.9%; $P < 0.001$]. This effect was comparable to that of salmon calcitonin (100 nM; 9.9 ± 1.1%; $P < 0.001$). In contrast, no inhibition of basal resorption was observed with equimolar concentrations of much shorter N-truncated PTH analogs, such as hPTH-(3–34) (300 nM; 18.7 ± 4.2%) or PTHrP-(7-36) (300 nM; 15.4 ± 4.9%), that bind as well or more effectively to the PTH1R as hPTH-(7-84).

Further, as shown in Fig. 2, hPTH-(7-84) (300 nM) also significantly inhibited (by 50% or more) agonist-induced bone resorption caused by a variety of osteotropic agents, including intact hPTH-(1-84) (3 nM), hPTH-(1-34) (3 nM), VitD (10 nM), PGE_2 (100 nM), and IL-11 (10 ng/ml). The antiresorptive effect of hPTH-(7-84) was dose dependent, with an IC_{50} of approximately 200 nM (data not shown). We considered the possibility that these inhibitory actions of hPTH-(7-84) might reflect antagonism, at the PTH1R, of an effect of locally secreted PTHrP to augment the responses to these other agonists. As shown in Fig. 3, however, neither hPTH-(3-34) (1 μM) nor PTHrP-(7-36) (1 μM), both of which act as PTH1R antagonists (see below) at the concentrations used, inhibited resorption induced by VitD (10 nM). With respect to a possible nonspecific or irreversible toxic effect of hPTH-(7-84), we observed that removal of the peptide after 24 h of exposure to calvariae did not impair the

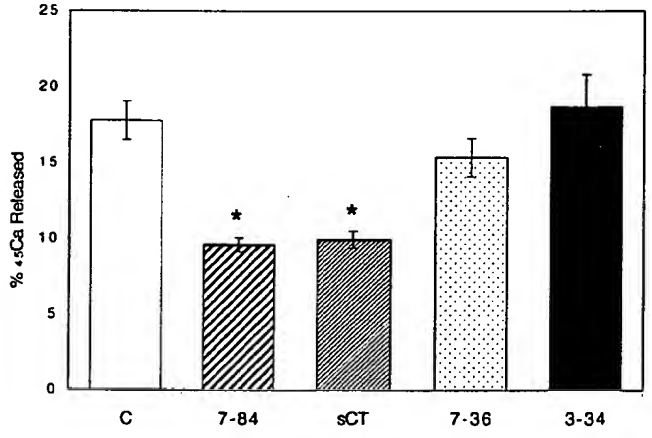

FIG. 1. Inhibition of basal bone resorption by hPTH-(7-84). Calvariae prelabeled with ^{45}Ca by maternal injection were isolated from neonatal mice as described in *Materials and Methods*. Bones were incubated individually, in treatment groups of four bones each, for 72 h after a single addition of vehicle alone (controls, C); hPTH-(7-84), hPTHrP-(7-36), or hPTH-(3-34) (all at 300 nM); or salmon calcitonin (sCT; 100 nM). Results are expressed as percentages of total ^{45}Ca released over 72 h. Values shown are the mean ± SEM of results from several (three to six) independent experiments. *, $P < 0.001$ *vs.* controls.

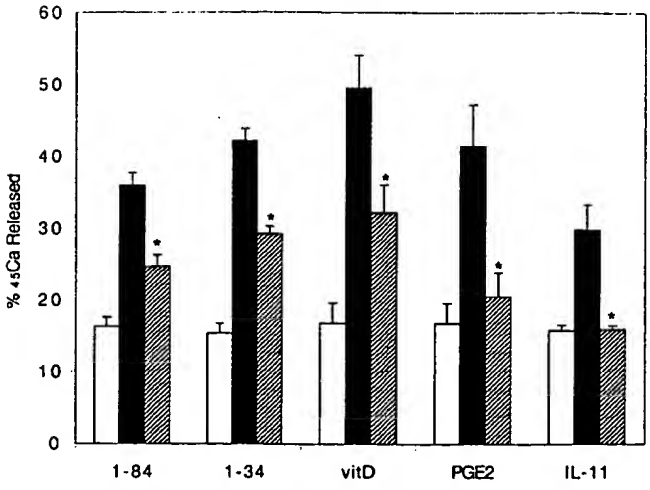

FIG. 2. Inhibition by hPTH-(7-84) of bone resorption induced by various osteotropic agents. Resorption assays were conducted as described in Fig. 1 for controls (□), osteotropic agents alone (■), or osteotropic agents in combination with 300 nM hPTH-(7-84) (▨). Osteotropic agents were employed at the following concentrations: hPTH-(1-84), 3 nM; hPTH-(1-34), 3 nM; VitD, 10 nM; PGE_2, 100 nM; and IL-11, 10 ng/ml. Results are expressed as the mean ± SEM of quadruplicate determinations. Each experiment was repeated three times. *, $P < 0.05$ for difference between osteotropic agent alone *vs.* osteotropic agent plus hPTH-(7-84).

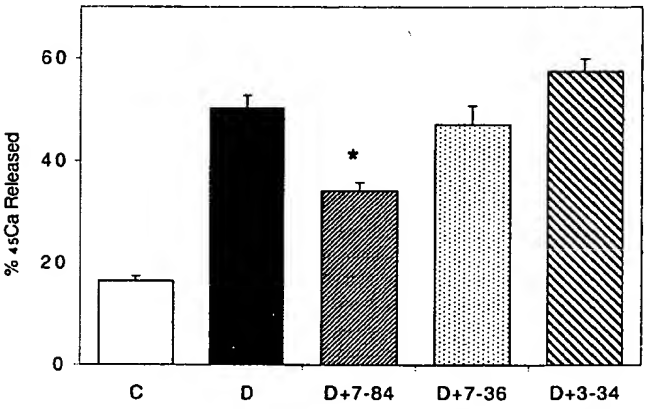

FIG. 3. Inhibition of VitD-induced bone resorption by hPTH-(7-84), but not by hPTHrP-(7-36) or hPTH-(3-34). Calvarial bones, incubated as described in Fig. 1, were treated with vehicle alone (C) or with 10 nM VitD, either alone (D) or together with 300 nM hPTH-(7-84) (D + 7-84), 1 μM hPTHrP-(7-36) (D + 7-36), or 1 μM hPTH-(3-34) (D + 3-34). Results are expressed as the mean ± SEM of quadruplicate determinations and are representative of three independent experiments. *, $P < 0.05$ between VitD alone and VitD plus PTH-(7-84).

resorptive response of the bones to subsequently added VitD (data not shown).

hPTH-(7-84) does not activate adenylyl cyclase or measurably inhibit the binding of a radiolabeled hPTH-(1-34) analog to PTH1Rs expressed on ROS 17/2.8 rat osteosarcoma cells, which also express CPTHRs (21, 27). To directly address the possibility that hPTH-(7-84) nevertheless might antagonize signaling by PTH1R agonists, either directly at the PTH1R or via activation of CPTHRs, ROS 17/2.8 cells were incubated with hPTH-(1-34) at a submaximal concentration (3 nM) in the absence or presence of excess hPTH-(7-84) (0.1–1 μM). As shown in Fig. 4, we observed no inhibition of the cAMP response to PTH-(1-34) by hPTH-(7-84) (0.1–1 μM), whereas both PTHrP-(7-36) and PTH-(3-34), when present at 1 μM, inhibited the response by 50%. In related experiments undiluted samples of conditioned medium from calvarial resorption assays (described above) to which 300 nM hPTH-(7-84), PTHrP-(7-36) or PTH-(3-34) had been added 72 h earlier also were tested for inhibition of hPTH-(1-34)-induced cAMP accumulation in ROS 17/2.8 cells. In none of these samples was inhibition of the cAMP response to hPTH-(1-34) observed (data not shown).

Inhibition of bone resorption by hPTH-(7-84) could result from decreased osteoclast formation, inhibition of the activity or survival of mature osteoclasts, or both. To determine whether hPTH-(7-84) impairs osteoclast formation, the activity of this fragment was studied in cultures of whole murine bone marrow. As shown in Fig. 5A, hPTH-(7-84) alone exerted no effect on the formation of TRAP+MNCs, although basal osteoclast formation in this system is low, and an inhibitory effect therefore might not be easily detectable. On the other hand, when osteoclast formation was stimulated by VitD (10 nM), hPTH-(7-84) (300 nM) caused a striking (70%) reduction in the formation of TRAP+MNCs relative to the effect of VitD alone [VitD, 153 ± 38 cells; VitD + hPTH-(7-84), 53 ± 14 cells]. In contrast,

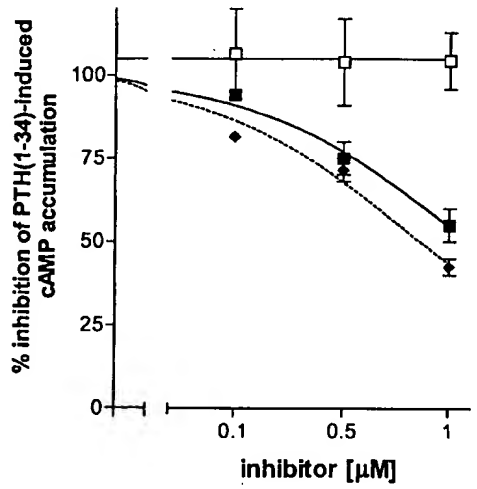

FIG. 4. Inhibition of cAMP accumulation in PTH-treated ROS 17/2.8 cells. Cells were stimulated with an approximately half-maximal concentration of PTH-(1-34) (3 nM) in the absence or presence of increasing concentrations of PTH-(7-84) (□, *solid line*), PTH-(3-34) (♦, *dashed line*), or PTHrP-(7-36) (■, *solid line*). Data are expressed as percentages of the cAMP response to 3 nM PTH-(1-34) alone and represent the results (mean ± SEM) of at least two independent experiments.

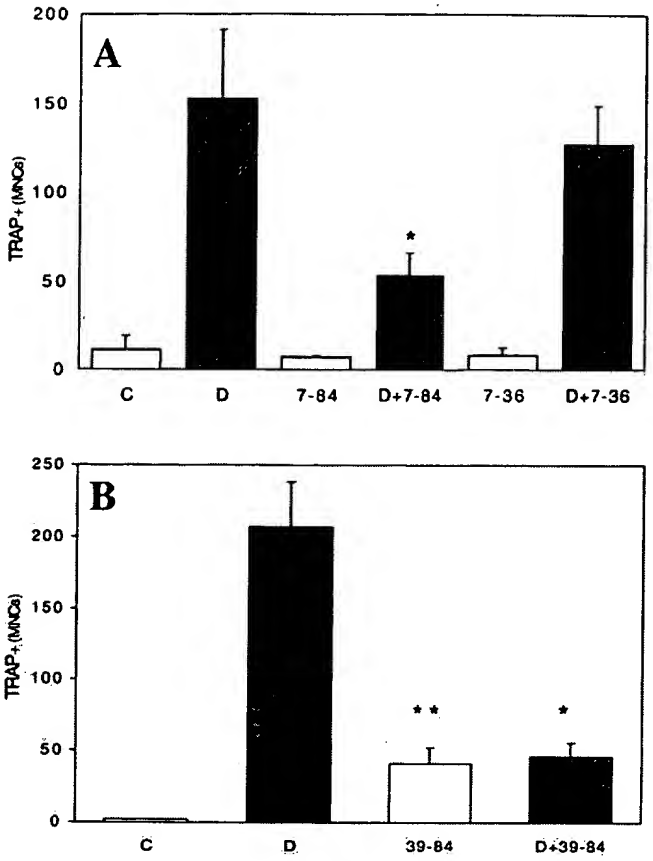

FIG. 5. Inhibition of osteoclast generation by hPTH-(7-84) and hPTH-(39-84). Whole bone marrow was isolated and cultured as described in *Materials and Methods*. Adherent and nonadherent cells were maintained in culture for 12 d, and the indicated treatments were added three times weekly, as described in *Materials and Methods*. At the end of the culture period, cells were fixed and stained for TRAP. A: C, Control; D, VitD (10 nM); 7-84, hPTH-(7-84) (300 nM); 7-36, hPTHrP-(7-36) (300 nM). B: C, Control; D, VitD (10 nM); 39-84, hPTH-(39-84) (3000 nM). Values (number of cells per well) are expressed as the mean ± SD for triplicate determinations (see *Materials and Methods*). Experiments were repeated twice. *, $P < 0.05$ *vs.* VitD alone; **, $P < 0.05$ *vs.* control.

PTHrP-(7-36) did not inhibit TRAP+MNC formation induced by VitD [VitD + PTHrP-(7-36), 127 ± 22 cells; Fig. 5A]. To determine whether shorter C-terminal PTH fragments could regulate osteoclast formation, we tested the effects of hPTH-(39-84), alone or in combination with VitD (10 nM). As shown in Fig. 5B, hPTH-(39-84) alone, at 3000 nM, slightly stimulated osteoclast formation (41 ± 11 cells), as reported previously for short CPTH fragments (24, 33). Like hPTH-(7-84), however, hPTH-(39-84) dramatically inhibited osteoclast formation promoted by VitD [VitD, 207 ± 31 cells; VitD + hPTH-(39-84), 46 ± 10 cells].

Discussion

Recent studies demonstrating that the extended CPTH fragment hPTH-(7-84) can inhibit the calcemic effects of PTH-(1-84) and PTH-(1-34) in thyroparathyroidectomized animals have suggested that CPTH peptides, normally present in blood and previously assumed to be biologically inert prod-

ucts of PTH metabolism, may be physiologically active (20, 21). As the hypocalcemic actions of hPTH-(7-84) described *in vivo* were associated with lowering of serum phosphate but were not accompanied by significant changes in urinary calcium or phosphate excretion (20, 21), a primary effect of this CPTH peptide on bone seems likely. The present *in vitro* studies were directed at clarifying whether hPTH-(7-84) might act directly on bone to inhibit the action of hPTH-(1-84) or hPTH-(1-34).

One possible mechanism for such an effect could involve direct antagonism by hPTH-(7-84) to prevent binding of hPTH-(1-34) or hPTH-(1-84) to PTH1Rs expressed on osteoblasts or marrow stromal cells. Because the antagonism *in vivo* is observed at much lower doses of hPTH-(7-84), relative to intact PTH, than that predicted to be necessary for direct antagonism at the PTH1R, however, we also considered the alternative possibility that hPTH-(7-84) may exert unique PTH1R-independent antiresorptive effects by activating CPTHRs expressed in bone cells. Our results are fully consistent with this latter hypothesis. Thus, we observed concentration-dependent inhibition of bone resorption in *ex vivo* calvarial organ cultures that was not mimicked by shorter, N-truncated PTH fragments that 1) are more effective PTH1R antagonists than is hPTH-(7-84) and 2) do not bind detectably to CPTHRs expressed on bone cells (28). Similar results were obtained in studies of osteoclastogenesis using whole bone marrow cultures, which further suggests that CPTHRs may be involved in the regulation of osteoclast formation. Because the number of mononuclear TRAP-positive cells formed in the marrow cultures also was reduced by hPTH-(7-84), the predominant action in osteoclastogenesis may be to inhibit formation of osteoclast precursors. The rapidity (1–2 h) of the hPTH-(7-84) effect observed *in vivo*, however, suggests that interference with the activity of mature osteoclasts also may be involved.

The antiresorptive effect of hPTH-(7-84) observed in the calvarial assay system contrasted sharply with the inability of hPTH-(3-34) or PTHrP-(7-36), introduced at similar concentrations, to inhibit resorption. Because both of these shorter, N-truncated peptides are effective *in vitro* PTH1R antagonists, whereas hPTH-(7-84) is not (as shown in Fig. 4), these results argue strongly against a mechanism involving direct antagonism by hPTH-(7-84) at the PTH1R of either endogenous PTHrP present within the cultured bones or exogenously added PTH. Moreover, the antiresorptive effect of hPTH-(7-84) *in vitro* was not restricted to resorption induced by added PTH, but was encountered in both control cultures and cultures treated with a variety of unrelated bone-resorbing agonists, including VitD, PGE_2 and IL-11. These findings point to a more generalized antiresorptive mechanism by which PTH-(7-84), presumably acting via CPTHRs, may limit the formation and, possibly, the activity of mature osteoclasts. This could reflect interference with the up-regulation of RANKL or macrophage colony-stimulating factor, the down-regulation of OPG, or both, that normally are triggered in marrow stromal cells and osteoblasts by these diverse resorbing agents (35). In this regard, we observed expression of CPTHRs by PTH1R-null osteoblasts and osteocytes (28) and by clonal marrow stromal cells that are capable of supporting PTH- or vitamin D-dependent osteoclast formation from hemopoietic progenitors *in vitro* (our unpublished observations). We also cannot yet exclude that the inhibition of resorption was mediated partly by a proapoptotic effect of hPTH-(7-84) on bone cells via activation of the CPTHR, as we previously reported in osteocytic cells (28). Moreover hPTH-(7-84) could act directly on mature osteoclasts, their hemopoietic precursors, or both to dampen cellular responsiveness to activation of RANK or c-*Fms* by their respective stromal cell or osteoblast-derived ligands. Indeed, evidence that shorter CPTH fragments alone can modestly induce osteoclast formation, as seen in the present study with hPTH-(39-84) and reported previously (24, 33), in contrast to the inhibitory effects of the same fragment upon osteoclast formation induced by vitamin D, points to a complexity in CPTHR action that is not readily explained at present, but that could involve disparate effects on distinct cell types involved in osteoclast formation. Direct analysis of CPTHR expression in such cells would be needed to address this possibility.

One prediction of our results might be that PTH-(1-84), which binds to CPTHRs with affinity comparable to that of PTH-(7-84) (21), should elicit less bone resorption than PTH-(1-34), which does not interact effectively with CPTHRs (28). Although few direct *in vitro* comparisons have been performed (24, 36), the available data do not indicate substantial or consistent differences in resorptive responses to these two peptides. Indeed, we observed in the calvarial resorption assay that the intact hormone reproducibly induced less ^{45}Ca release than did PTH-(1-34) at equimolar concentrations, although this difference was never statistically significant. This could indicate that when PTH1Rs and CPTHRs are exposed simultaneously to equimolar concentrations of a common ligand, the PTH1R-mediated resorptive response strongly predominates. Alternatively, it is possible that despite comparable binding affinity, intact PTH cannot activate CPTHRs as effectively as N-truncated peptides (by analogy with the disparate activation of PTH1Rs observed with PTH-(1-34) *vs.* PTH-(3-34)). On the other hand, because CPTH fragments normally circulate in plasma at concentrations at least 5- to 10-fold higher than those of intact PTH, a requirement for higher molar concentrations of CPTH ligands to activate CPTHRs might be expected. This concept is consistent with our finding that a 10- to 100-fold molar excess of CPTH ligand is needed to elicit functional antagonism of PTH1R-mediated resorption *in vitro*. The observation that hPTH-(7-84) could antagonize the calcemic response to hPTH-(1-84) at equimolar doses *in vivo* might be related to differential bioavailability or metabolism of the two peptides after their ip or iv administration (20, 21).

Secretion of CPTH fragments by the parathyroid glands is positively regulated by blood calcium (37). Thus, one possible physiological role of the antiresorptive action of N-truncated PTH fragments *in vivo* could be to modulate the extent of bone resorption induced by intact PTH in a manner responsive to the extracellular calcium concentration. Such a mechanism, for example, might allow for maximal release of calcium from bone only during severe hypocalcemia to supplement the ongoing renal and (indirect) intestinal actions of PTH. It is important to note that the chemical identities of all circulating CPTH fragments have not yet been completely defined. In particular, the existence in blood of PTH-(7-84) *per se* has not been directly demonstrated. On the other hand, the

recent immunochemical characterization of nonintact PTH peptides, which are especially abundant in renal failure, is consistent with the presence of extended CPTH fragments longer than those previously inferred from analyses of secreted or peripherally generated cleavage products, the N-termini of which ranged between positions 24 and 43 of the intact PTH sequence (19, 38). Thus, the possibility that PTH fragments similar or identical to PTH-(7-84) may be present in blood, especially in renal failure, at concentrations high enough to activate CPTHRs and thereby exert direct effects on bone resorption must be considered.

Acknowledgments

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* P.D. and M.R.J. contributed equally to this work.

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PA 9241 FSAMP

FORTEO™
teriparatide (rDNA origin) injection
750 mcg/3 mL

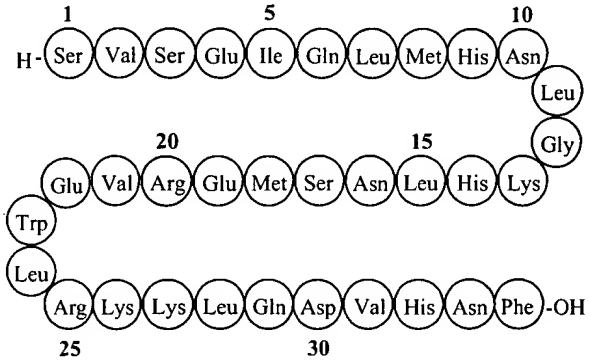
WARNING

In male and female rats, teriparatide caused an increase in the incidence of osteosarcoma (a malignant bone tumor) that was dependent on dose and treatment duration. The effect was observed at systemic exposures to teriparatide ranging from 3 to 60 times the exposure in humans given a 20-mcg dose. Because of the uncertain relevance of the rat osteosarcoma finding to humans, teriparatide should be prescribed only to patients for whom the potential benefits are considered to outweigh the potential risk. Teriparatide should not be prescribed for patients who are at increased baseline risk for osteosarcoma (including those with Paget's disease of bone or unexplained elevations of alkaline phosphatase, open epiphyses, or prior radiation therapy involving the skeleton) (*see* WARNINGS *and* PRECAUTIONS, Carcinogenesis).

DESCRIPTION

FORTEO™ [teriparatide (rDNA origin) injection] contains recombinant human parathyroid hormone (1-34), [rhPTH(1-34)], which has an identical sequence to the 34 N-terminal amino acids (the biologically active region) of the 84-amino acid human parathyroid hormone.

Teriparatide has a molecular weight of 4117.8 daltons and its amino acid sequence is shown below:



Teriparatide (rDNA origin) is manufactured by Eli Lilly and Company using a strain of *Escherichia coli* modified by recombinant DNA technology. FORTEO is supplied as a sterile, colorless, clear, isotonic solution in a glass cartridge which is pre-assembled into a disposable pen device for subcutaneous injection. Each prefilled delivery device is filled with 3.3 mL to deliver 3 mL. Each mL contains 250 mcg teriparatide (corrected for acetate, chloride, and water content), 0.41 mg glacial acetic acid, 0.10 mg sodium acetate (anhydrous), 45.4 mg mannitol, 3.0 mg Metacresol, and Water for Injection. In addition, hydrochloric acid solution 10% and/or sodium hydroxide solution 10% may have been added to adjust the product to pH 4.

Each cartridge pre-assembled into a pen device delivers 20 mcg of teriparatide per dose each day for up to 28 days.

See accompanying User Manual: Instructions for Use.

CLINICAL PHARMACOLOGY

Mechanism of Action

Endogenous 84-amino-acid parathyroid hormone (PTH) is the primary regulator of calcium and phosphate metabolism in bone and kidney. Physiological actions of PTH include regulation of bone metabolism, renal tubular reabsorption of calcium and phosphate, and intestinal calcium absorption. The biological actions of PTH and teriparatide are mediated through binding to specific high-affinity cell-surface receptors. Teriparatide and the 34 N-terminal amino acids of PTH bind to these receptors with the same affinity and have the same physiological actions on bone and kidney. Teriparatide is not expected to accumulate in bone or other tissues.

The skeletal effects of teriparatide depend upon the pattern of systemic exposure. Once-daily administration of teriparatide stimulates new bone formation on trabecular and cortical (periosteal and/or endosteal) bone surfaces by preferential stimulation of osteoblastic activity over osteoclastic activity. In monkey studies, teriparatide improved trabecular microarchitecture and increased bone mass and strength by stimulating new bone formation in both cancellous and cortical bone. In humans, the anabolic effects of teriparatide are manifest as an increase in skeletal mass, an increase in markers of bone formation and resorption, and an increase in bone strength. By contrast, continuous excess of endogenous PTH, as occurs in hyperparathyroidism, may be detrimental to the skeleton because bone resorption may be stimulated more than bone formation.

Human Pharmacokinetics

Teriparatide is extensively absorbed after subcutaneous injection; the absolute bioavailability is approximately 95% based on pooled data from 20-, 40-, and 80-mcg doses. The rates of absorption and elimination are rapid. The peptide reaches peak serum concentrations about 30 minutes after subcutaneous injection of a 20-mcg dose and declines to non-quantifiable concentrations within 3 hours.

Systemic clearance of teriparatide (approximately 62 L/hr in women and 94 L/hr in men) exceeds the rate of normal liver plasma flow, consistent with both hepatic and extra-hepatic clearance. Volume of distribution, following intravenous injection, is approximately 0.12 L/kg. Intersubject variability in systemic clearance and volume of distribution is 25% to 50%. The half-life of teriparatide in serum is 5 minutes when administered by intravenous injection and approximately 1 hour when administered by subcutaneous injection. The longer half-life following subcutaneous administration reflects the time required for absorption from the injection site.

No metabolism or excretion studies have been performed with teriparatide. However, the mechanisms of metabolism and elimination of PTH(1-34) and intact PTH have been extensively described in published literature. Peripheral metabolism of PTH is believed to occur by non-specific enzymatic mechanisms in the liver followed by excretion via the kidneys.

Special Populations

Pediatric — Pharmacokinetic data in pediatric patients are not available (*see* WARNINGS).

Geriatric — No age-related differences in teriparatide pharmacokinetics were detected (range 31 to 85 years).

Gender — Although systemic exposure to teriparatide was approximately 20% to 30% lower in men than women, the recommended dose for both genders is 20 mcg/day.

Race — The populations included in the pharmacokinetic analyses were 98.5% Caucasian. The influence of race has not been determined.

Renal insufficiency — No pharmacokinetic differences were identified in 11 patients with mild or moderate renal insufficiency [creatinine clearance (CrCl) 30 to 72 mL/min] administered a single dose of teriparatide. In 5 patients with severe renal insufficiency (CrCl<30 mL/min), the

AUC and $T_{1/2}$ of teriparatide were increased by 73% and 77%, respectively. Maximum serum concentration of teriparatide was not increased. No studies have been performed in patients undergoing dialysis for chronic renal failure (*see* PRECAUTIONS).

Heart failure — No clinically relevant pharmacokinetic, blood pressure, or pulse rate differences were identified in 13 patients with stable New York Heart Association Class I to III heart failure after the administration of two 20-mcg doses of teriparatide.

Hepatic insufficiency — Non-specific proteolytic enzymes in the liver (possibly Kupffer cells) cleave PTH(1-34) and PTH(1-84) into fragments that are cleared from the circulation mainly by the kidney. No studies have been performed in patients with hepatic impairment.

Drug Interactions

Hydrochlorothiazide — In a study of 20 healthy people, the coadministration of hydrochlorothiazide 25 mg with teriparatide did not affect the serum calcium response to teriparatide 40 mcg. The 24-hour urine excretion of calcium was reduced by a clinically unimportant amount (15%). The effect of coadministration of a higher dose of hydrochlorothiazide with teriparatide on serum calcium levels has not been studied.

Furosemide — In a study of 9 healthy people and 17 patients with mild, moderate, or severe renal insufficiency (CrCl 13 to 72 mL/min), coadministration of intravenous furosemide (20 to 100 mg) with teriparatide 40 mcg resulted in small increases in the serum calcium (2%) and 24-hour urine calcium (37%) responses to teriparatide that did not appear to be clinically important.

Human Pharmacodynamics

Effects on mineral metabolism

Teriparatide affects calcium and phosphorus metabolism in a pattern consistent with the known actions of endogenous PTH (eg, increases serum calcium and decreases serum phosphorus).

Serum calcium concentrations

When teriparatide 20 mcg is administered once daily, the serum calcium concentration increases transiently, beginning approximately 2 hours after dosing and reaching a maximum concentration between 4 and 6 hours (median increase, 0.4 mg/dL). The serum calcium concentration begins to decline approximately 6 hours after dosing and returns to baseline by 16 to 24 hours after each dose.

In a clinical study of postmenopausal women with osteoporosis, the median peak serum calcium concentration measured 4 to 6 hours after dosing with FORTEO (teriparatide 20 mcg) was 2.42 mmol/L (9.68 mg/dL) at 12 months. The peak serum calcium remained below 2.76 mmol/L (11.0 mg/dL) in >99% of women at each visit. Sustained hypercalcemia was not observed.

In this study, 11.1% of women treated with FORTEO had at least 1 serum calcium value above the upper limit of normal [2.64 mmol/L (10.6 mg/dL)] compared with 1.5% of women treated with placebo. The percentage of women treated with FORTEO whose serum calcium was above the upper limit of normal on consecutive 4- to 6-hour post-dose measurements was 3.0% compared with 0.2% of women treated with placebo. In these women, calcium supplements and/or FORTEO doses were reduced. The timing of these dose reductions was at the discretion of the investigator. FORTEO dose adjustments were made at varying intervals after the first observation of increased serum calcium (median 21 weeks). During these intervals, there was no evidence of progressive increases in serum calcium.

In a clinical study of men with either primary or hypogonadal osteoporosis, the effects on serum calcium were similar to those observed in postmenopausal women. The median peak serum calcium concentration measured 4 to 6 hours after dosing with FORTEO was 2.35 mmol/L

(9.44 mg/dL) at 12 months. The peak serum calcium remained below 2.76 mmol/L (11.0 mg/dL) in 98% of men at each visit. Sustained hypercalcemia was not observed.

In this study, 6.0% of men treated with FORTEO daily had at least 1 serum calcium value above the upper limit of normal [2.64 mmol/L (10.6 mg/dL)] compared with none of the men treated with placebo. The percentage of men treated with FORTEO whose serum calcium was above the upper limit of normal on consecutive measurements was 1.3% (2 men) compared with none of the men treated with placebo. Although calcium supplements and/or FORTEO doses could have been reduced in these men, only calcium supplementation was reduced (*see* PRECAUTIONS *and* ADVERSE EVENTS).

In a clinical study of women previously treated for 18 to 39 months with raloxifene (n=26) or alendronate (n=33), mean serum calcium >12 hours after FORTEO injection was increased by 0.09 to 0.14 mmol/L (0.36 to 0.56 mg/dL), after 1 to 6 months of FORTEO treatment compared with baseline. Of the women pretreated with raloxifene, 3 (11.5%) had a serum calcium >2.76 mmol/L (11.0 mg/dL), and of those pretreated with alendronate, 3 (9.1%) had a serum calcium >2.76 mmol/L (11.0 mg/dL). The highest serum calcium reported was 3.12 mmol/L (12.5 mg/dL). None of the women had symptoms of hypercalcemia. There were no placebo controls in this study.

Urinary calcium excretion

In a clinical study of postmenopausal women with osteoporosis who received 1000 mg of supplemental calcium and at least 400 IU of vitamin D, daily FORTEO increased urinary calcium excretion. The median urinary excretion of calcium was 4.8 mmol/day (190 mg/day) at 6 months and 4.2 mmol/day (170 mg/day) at 12 months. These levels were 0.76 mmol/day (30 mg/day) and 0.30 mmol/day (12 mg/day) higher, respectively, than in women treated with placebo. The incidence of hypercalciuria (>7.5 mmol Ca/day or 300 mg/day) was similar in the women treated with FORTEO or placebo.

In a clinical study of men with either primary or hypogonadal osteoporosis who received 1000 mg of supplemental calcium and at least 400 IU of vitamin D, daily FORTEO had inconsistent effects on urinary calcium excretion. The median urinary excretion of calcium was 5.6 mmol/day (220 mg/day) at 1 month and 5.3 mmol/day (210 mg/day) at 6 months. These levels were 0.50 mmol/day (20 mg/day) higher and 0.20 mmol/day (8.0 mg/day) lower, respectively, than in men treated with placebo. The incidence of hypercalciuria (>7.5 mmol Ca/day or 300 mg/day) was similar in the men treated with FORTEO or placebo.

Phosphorus and vitamin D

In single-dose studies, teriparatide produced transient phosphaturia and mild transient reductions in serum phosphorus concentration. However, hypophosphatemia (<0.74 mmol/L or 2.4 mg/dL) was not observed in clinical trials with FORTEO.

In clinical trials of daily FORTEO, the median serum concentration of 1,25-dihydroxyvitamin D was increased at 12 months by 19% in women and 14% in men, compared with baseline. In the placebo group, this concentration decreased by 2% in women and increased by 5% in men. The median serum 25-hydroxyvitamin D concentration at 12 months was decreased by 19% in women and 10% in men compared with baseline. In the placebo group, this concentration was unchanged in women and increased by 1% in men.

Effects on markers of bone turnover

Daily administration of FORTEO to men and postmenopausal women with osteoporosis in clinical studies stimulated bone formation, as shown by increases in the formation markers serum bone-specific alkaline phosphatase (BSAP) and procollagen I carboxy-terminal propeptide (PICP). Data on biochemical markers of bone turnover were available for the first 12 months of treatment. Peak concentrations of PICP at 1 month of treatment were approximately 41% above baseline, followed by a decline to near-baseline values by 12 months. BSAP concentrations

increased by 1 month of treatment and continued to rise more slowly from 6 through 12 months. The maximum increases of BSAP were 45% above baseline in women and 23% in men. After discontinuation of therapy, BSAP concentrations returned toward baseline. The increases in formation markers were accompanied by secondary increases in the markers of bone resorption: urinary N-telopeptide (NTX) and urinary deoxypyridinoline (DPD), consistent with the physiological coupling of bone formation and resorption in skeletal remodeling. Changes in BSAP, NTX, and DPD were lower in men than in women, possibly because of lower systemic exposure to teriparatide in men.

CLINICAL STUDIES

Treatment of Osteoporosis in Postmenopausal Women

The safety and efficacy of once-daily FORTEO, median exposure of 19 months, were examined in a double-blind, placebo-controlled clinical study of 1637 postmenopausal women with osteoporosis (FORTEO 20 mcg, n=541).

This multicenter study was performed in the US and 16 other countries. All women received 1000 mg of calcium per day and at least 400 IU of vitamin D per day. Baseline and endpoint spinal radiographs were evaluated using the semiquantitative scoring method of Genant et al [*J Bone Miner Res* 1993;8(9):1137-48]. Ninety percent of the women in the study had 1 or more radiographically diagnosed vertebral fractures at baseline. The primary efficacy endpoint was the occurrence of new radiographically diagnosed vertebral fractures defined as changes in the height of previously undeformed vertebrae. Such fractures are not necessarily symptomatic.

Effect on fracture incidence

New vertebral fractures — FORTEO, when taken with calcium and vitamin D and compared with calcium and vitamin D alone, reduced the risk of 1 or more new vertebral fractures from 14.3% of women in the placebo group to 5.0% in the FORTEO group. This difference was statistically significant ($p<0.001$); the absolute reduction in risk was 9.3% and the relative reduction was 65%. FORTEO was effective in reducing the risk for vertebral fractures regardless of age, baseline rate of bone turnover, or baseline BMD.

Table 1. Effect of FORTEO on Risk of Vertebral Fractures in Postmenopausal Women with Osteoporosis

	Percent of Women With Fracture			
	FORTEO (N=444)	Placebo (N=448)	Absolute Risk Reduction (%, 95% CI)	Relative Risk Reduction (%, 95% CI)
New fracture (≥1)	5.0[a]	14.3	9.3 (5.5-13.1)	65 (45-78)
1 fracture	3.8	9.4		
2 fractures	0.9	2.9		
≥3 fractures	0.2	2.0		

[a] $p\leq0.001$ compared with placebo.

New nonvertebral osteoporotic fractures — Table 2 shows the effect of FORTEO on the risk of nonvertebral fractures. FORTEO significantly reduced the risk of any nonvertebral fracture from 5.5% in the placebo group to 2.6% in the FORTEO group ($p<0.05$). The absolute reduction in risk was 2.9% and the relative reduction was 53%.

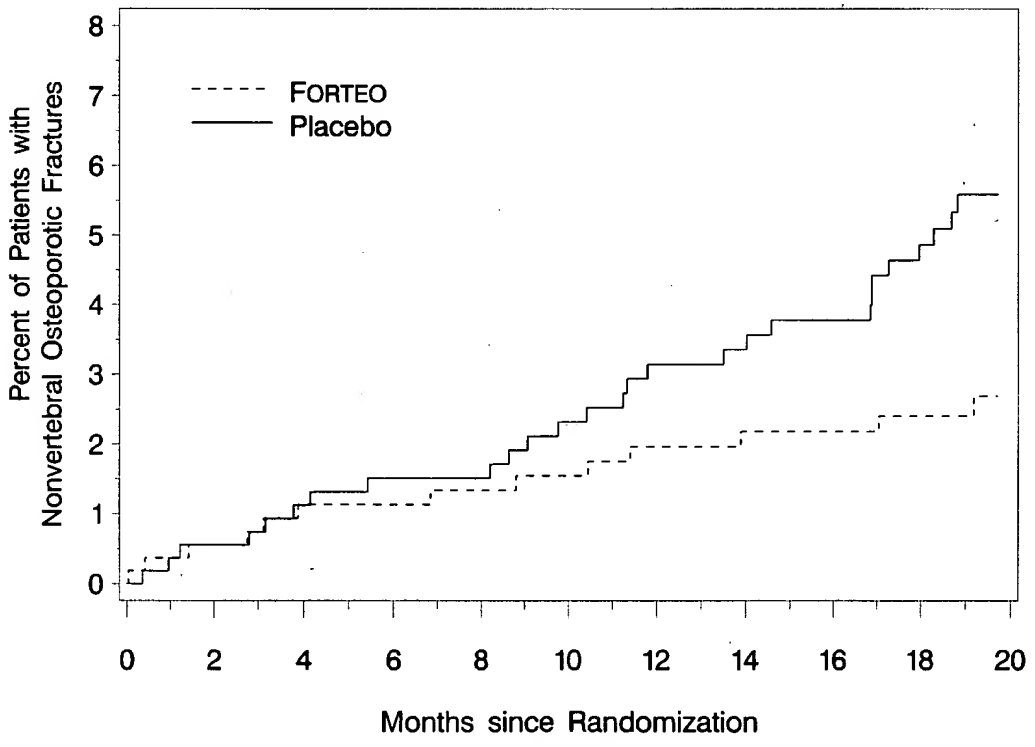
Table 2. Effects of FORTEO on Risk of New Nonvertebral Fractures in Postmenopausal Women with Osteoporosis

	FORTEO[a] N=541	Placebo[a] N=544
Skeletal site		
Wrist	2 (0.4%)	7 (1.3%)
Ribs	3 (0.6%)	5 (0.9%)
Hip	1 (0.2%)	4 (0.7%)
Ankle/Foot	1 (0.2%)	4 (0.7%)
Humerus	2 (0.4%)	2 (0.4%)
Pelvis	0	3 (0.6%)
Other	6 (1.1%)	8 (1.5%)
Total	14 (2.6%)[b]	30 (5.5%)

[a] Data shown as number (%) of women with fractures.
[b] $p<0.05$ compared with placebo.

The cumulative percentage of postmenopausal women with osteoporosis who sustained new nonvertebral fractures was lower in women treated with FORTEO than in women treated with placebo (*see* Figure 1).

Figure 1. Cumulative percentage of postmenopausal women with osteoporosis sustaining new nonvertebral osteoporotic fractures.*



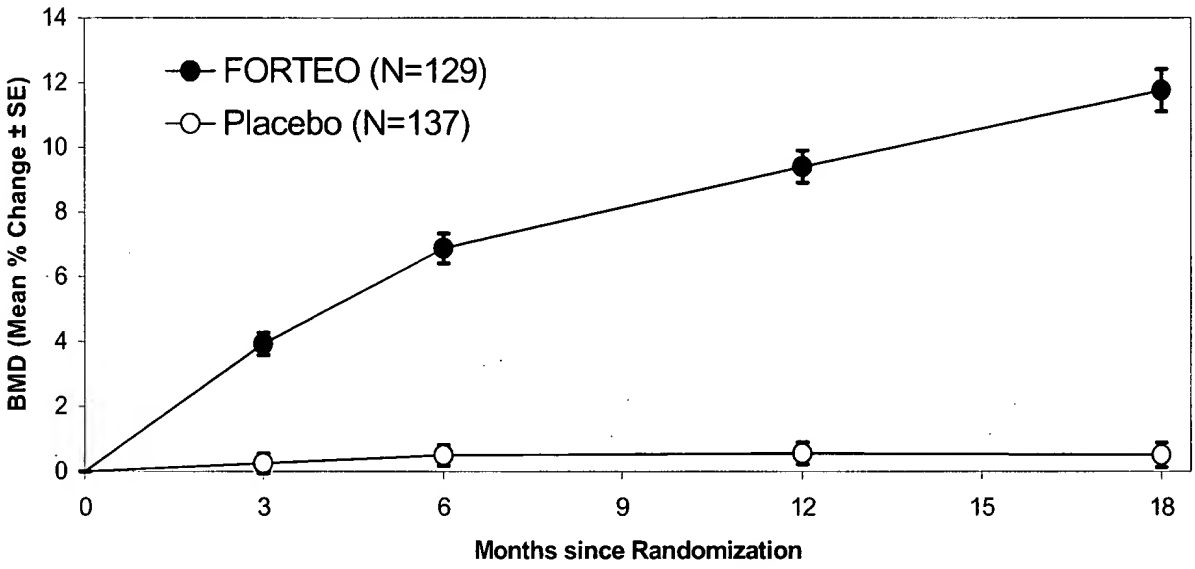
* This graph includes all fractures listed above in Table 2.

Effect on bone mineral density (BMD)

FORTEO increased lumbar spine BMD in postmenopausal women with osteoporosis. Statistically significant increases were seen at 3 months and continued throughout the treatment period, as shown in Figure 2.

Figure 2. Time course of change in lumbar spine BMD in postmenopausal women with osteoporosis treated with FORTEO vs placebo (women with data available at all time points).

($p<0.001$ for FORTEO compared with placebo at each post-baseline time point)



Postmenopausal women with osteoporosis who were treated with FORTEO also had statistically significant increases in BMD at the femoral neck, total hip, and total body (*see* Table 3).

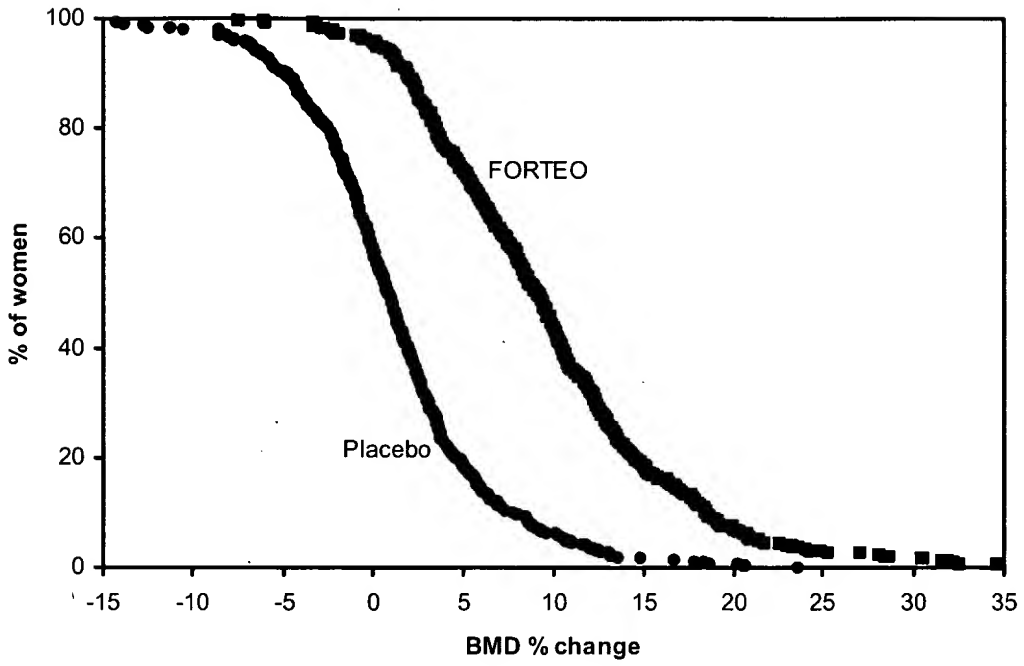
Table 3. Mean Percent Change in BMD from Baseline to Endpoint* in Postmenopausal Women with Osteoporosis, Treated with FORTEO or Placebo

	FORTEO N=541	Placebo N=544
Lumbar spine BMD	9.7[a]	1.1
Femoral neck BMD	2.8[b]	-0.7
Total hip BMD	2.6[b]	-1.0
Trochanter BMD	3.5[b]	-0.2
Intertrochanter BMD	2.6[b]	-1.3
Ward's triangle BMD	4.2[b]	-0.8
Total body BMD	0.6[b]	-0.5
Distal 1/3 radius BMD	-2.1	-1.3
Ultradistal radius BMD	-0.1	-1.6

* Intent-to-treat analysis, last observation carried forward.
[a] $p<0.001$ compared with placebo.
[b] $p<0.05$ compared with placebo.

Figure 3 shows the cumulative distribution of the percentage change from baseline of lumbar spine BMD for the FORTEO and placebo groups. FORTEO treatment increased lumbar spine BMD from baseline in 96% of postmenopausal women treated (*see* Figure 3). Seventy-two percent of patients treated with FORTEO achieved at least a 5% increase in spine BMD, and 44% gained 10% or more.

Figure 3. Percent of postmenopausal women with osteoporosis attaining a lumbar spine BMD percent change from baseline at least as great as the value on the x-axis (median duration of treatment 19 months).



Both treatment groups lost height during the trial. The mean decreases were 3.61 and 2.81 mm in the placebo and FORTEO groups, respectively.

Bone histology — The effects of teriparatide on bone histology were evaluated in iliac crest biopsies of 35 postmenopausal women treated for 12 to 24 months with calcium and vitamin D and teriparatide 20 or 40 mcg/day. Normal mineralization was observed with no evidence of cellular toxicity. The new bone formed with teriparatide was of normal quality (as evidenced by the absence of woven bone and marrow fibrosis).

Treatment to increase bone mass in men with primary or hypogonadal osteoporosis — The safety and efficacy of once-daily FORTEO, median exposure of 10 months, were examined in a double-blind, placebo-controlled clinical study of 437 men with either primary (idiopathic) or hypogonadal osteoporosis (FORTEO 20 mcg, n=151). This multicenter efficacy study was performed in the US and 10 other countries. All men received 1000 mg of calcium per day and at least 400 IU of vitamin D per day. The primary efficacy endpoint was change in lumbar spine BMD.

FORTEO increased lumbar spine BMD in men with primary or hypogonadal osteoporosis. Statistically significant increases were seen at 3 months and continued throughout the treatment period. FORTEO was effective in increasing lumbar spine BMD regardless of age, baseline rate of bone turnover, and baseline BMD. The effects of FORTEO at additional skeletal sites are shown in Table 4.

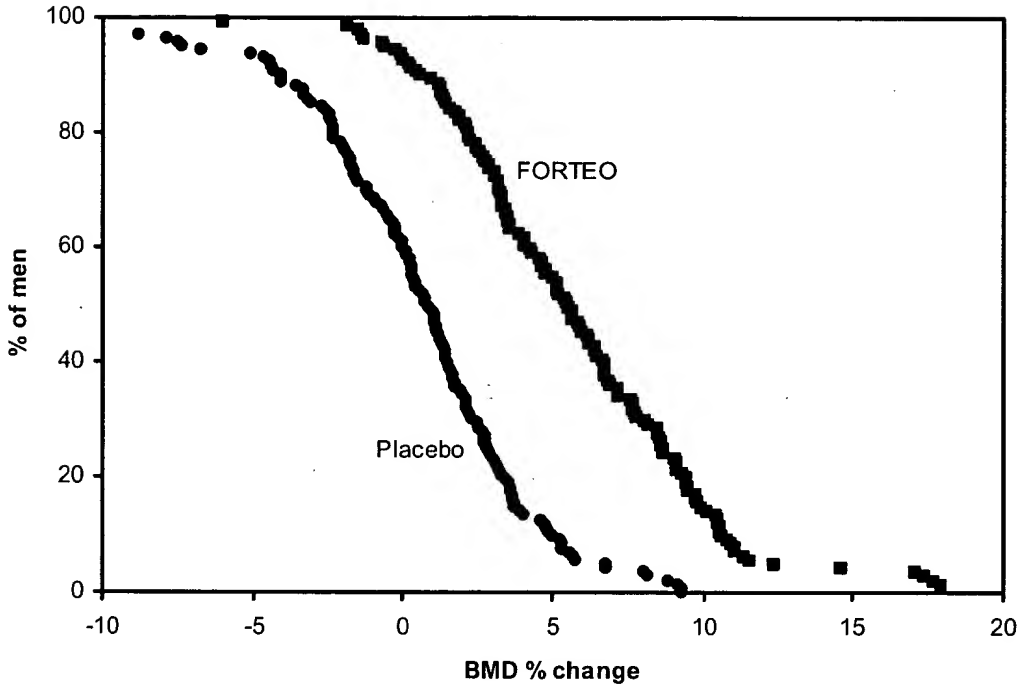
Table 4. Mean Percent Change in BMD from Baseline to Endpoint* in Men with Primary or Hypogonadal Osteoporosis, Treated with FORTEO or Placebo for a Median of 10 Months

	FORTEO N=151	Placebo N=147
Lumbar spine BMD	5.9[a]	0.5
Femoral neck BMD	1.5[b]	0.3
Total hip BMD	1.2	0.5
Trochanter BMD	1.3	1.1
Intertrochanter BMD	1.2	0.6
Ward's triangle BMD	2.8	1.1
Total body BMD	0.4	-0.4
Distal 1/3 radius BMD	-0.5	-0.2
Ultradistal radius BMD	-0.5	-0.3

* Intent-to-treat analysis, last observation carried forward.
[a] $p<0.001$ compared with placebo.
[b] $p<0.05$ compared with placebo.

Figure 4 shows the cumulative distribution of the percentage change from baseline of lumbar spine BMD for the FORTEO and placebo groups. FORTEO treatment for a median of 10 months increased lumbar spine BMD from baseline in 94% of men treated. Fifty-three percent of patients treated with FORTEO achieved at least a 5% increase in spine BMD, and 14% gained 10% or more.

Figure 4. Percent of men with primary or hypogonadal osteoporosis attaining a lumbar spine BMD percent change from baseline at least as great as the value on the x-axis (median duration of treatment 10 months).



INDICATIONS AND USAGE

FORTEO is indicated for the treatment of postmenopausal women with osteoporosis who are at high risk for fracture. These include women with a history of osteoporotic fracture, or who have multiple risk factors for fracture, or who have failed or are intolerant of previous osteoporosis therapy, based upon physician assessment (*see* BLACK BOX WARNING). In postmenopausal women with osteoporosis, FORTEO increases BMD and reduces the risk of vertebral and nonvertebral fractures.

FORTEO is indicated to increase bone mass in men with primary or hypogonadal osteoporosis who are at high risk for fracture. These include men with a history of osteoporotic fracture, or who have multiple risk factors for fracture, or who have failed or are intolerant to previous osteoporosis therapy, based upon physician assessment (*see* BLACK BOX WARNING). In men with primary or hypogonadal osteoporosis, FORTEO increases BMD. The effects of FORTEO on risk for fracture in men have not been studied.

- FORTEO reduces the risk of vertebral fractures in postmenopausal women with osteoporosis.
- FORTEO reduces the risk of nonvertebral fractures in postmenopausal women with osteoporosis.
- FORTEO increases vertebral and femoral neck BMD in postmenopausal women with osteoporosis and in men with primary or hypogonadal osteoporosis.
- The effects of FORTEO on fracture risk have not been studied in men.

CONTRAINDICATIONS

FORTEO should not be given to patients with hypersensitivity to teriparatide or to any of its excipients.

WARNINGS

In male and female rats, teriparatide caused an increase in the incidence of osteosarcoma (a malignant bone tumor) that was dependent on dose and treatment duration (*see* BLACK BOX WARNING *and* PRECAUTIONS; Carcinogenesis).

The following categories of patients have increased baseline risk of osteosarcoma and therefore should not be treated with FORTEO:

- Paget's disease of bone. FORTEO should not be given to patients with Paget's disease of bone. Unexplained elevations of alkaline phosphatase may indicate Paget's disease of bone.
- Pediatric populations. FORTEO has not been studied in pediatric populations. FORTEO should not be used in pediatric patients or young adults with open epiphyses.
- Prior radiation therapy. Patients with a prior history of radiation therapy involving the skeleton should be excluded from treatment with FORTEO.

Patients with bone metastases or a history of skeletal malignancies should be excluded from treatment with FORTEO.

Patients with metabolic bone diseases other than osteoporosis should be excluded from treatment with FORTEO.

FORTEO has not been studied in patients with pre-existing hypercalcemia. These patients should be excluded from treatment with FORTEO because of the possibility of exacerbating hypercalcemia.

PRECAUTIONS

General

The safety and efficacy of FORTEO have not been evaluated beyond 2 years of treatment. Consequently, use of the drug for more than 2 years is not recommended.

In clinical trials, the frequency of urolithiasis was similar in patients treated with FORTEO and placebo. However, FORTEO has not been studied in patients with active urolithiasis. If active urolithiasis or pre-existing hypercalciuria are suspected, measurement of urinary calcium excretion should be considered. FORTEO should be used with caution in patients with active or recent urolithiasis because of the potential to exacerbate this condition.

Hypotension

In short-term clinical pharmacology studies with teriparatide, transient episodes of symptomatic orthostatic hypotension were observed infrequently. Typically, an event began within 4 hours of dosing and spontaneously resolved within a few minutes to a few hours. When transient orthostatic hypotension occurred, it happened within the first several doses, it was relieved by placing the person in a reclining position, and it did not preclude continued treatment.

Concomitant treatment with digitalis

In a study of 15 healthy people administered digoxin daily to steady state, a single FORTEO dose did not alter the effect of digoxin on the systolic time interval (from electrocardiographic Q-wave onset to aortic valve closure, a measure of digoxin's calcium-mediated cardiac effect). However, sporadic case reports have suggested that hypercalcemia may predispose patients to digitalis toxicity. Because FORTEO transiently increases serum calcium, FORTEO should be used with caution in patients taking digitalis.

Hepatic, renal, and cardiac

Limited information is available to evaluate safety in patients with hepatic, renal, and cardiac disease.

Information for Patients

For safe and effective use of FORTEO, the physician should inform patients about the following:

General

Patients should read the *Medication Guide* and pen *User Manual* before starting therapy with FORTEO and re-read them each time the prescription is renewed.

Osteosarcomas in rats

Patients should be made aware that FORTEO caused osteosarcomas in rats and that the clinical relevance of these findings is unknown.

Orthostatic hypotension

FORTEO should be administered initially under circumstances where the patient can immediately sit or lie down if symptoms occur. Patients should be instructed that if they feel lightheaded or have palpitations after the injection, they should sit or lie down until the symptoms resolve. If symptoms persist or worsen, patients should be instructed to consult a physician before continuing treatment (*see* PRECAUTIONS, General).

Hypercalcemia

Although symptomatic hypercalcemia was not observed in clinical trials, physicians should instruct patients to contact a health care provider if they develop persistent symptoms of hypercalcemia (ie, nausea, vomiting, constipation, lethargy, muscle weakness).

Use of the pen

Patients should be instructed on how to properly use the delivery device (refer to *User Manual*), properly dispose of needles, and be advised not to share their pens with other patients.

Other osteoporosis treatments

Patients should be informed regarding the roles of supplemental calcium and/or vitamin D, weight-bearing exercise, and modification of certain behavioral factors such as cigarette smoking and/or alcohol consumption.

Laboratory Tests

Serum calcium — FORTEO transiently increases serum calcium, with the maximal effect observed at approximately 4 to 6 hours post-dose. By 16 hours post-dose, serum calcium generally has returned to or near baseline. These effects should be kept in mind because serum calcium concentrations observed within 16 hours after a dose may reflect the pharmacologic effect of teriparatide. Persistent hypercalcemia was not observed in clinical trials with FORTEO. If persistent hypercalcemia is detected, treatment with FORTEO should be discontinued pending further evaluation of the cause of hypercalcemia.

Patients known to have an underlying hypercalcemic disorder, such as primary hyperparathyroidism, should not be treated with FORTEO (*see* WARNINGS).

Urinary calcium — FORTEO increases urinary calcium excretion, but the frequency of hypercalciuria in clinical trials was similar for patients treated with FORTEO and placebo (*see* CLINICAL PHARMACOLOGY, Human Pharmacodynamics).

Renal function — No clinically important adverse renal effects were observed in clinical studies. Assessments included creatinine clearance; measurements of blood urea nitrogen (BUN), creatinine, and electrolytes in serum; urine specific gravity and pH; and examination of urine sediment. Long-term evaluation of patients with severe renal insufficiency, patients undergoing acute or chronic dialysis, or patients who have functioning renal transplants has not been performed.

Serum uric acid — FORTEO increases serum uric acid concentrations. In clinical trials, 2.8% of FORTEO patients had serum uric acid concentrations above the upper limit of normal compared with 0.7% of placebo patients. However, the hyperuricemia did not result in an increase in gout, arthralgia, or urolithiasis.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenesis

Two carcinogenicity bioassays were conducted in Fischer 344 rats. In the first study, male and female rats were given daily subcutaneous teriparatide injections of 5, 30, or 75 mcg/kg/day for 24 months from 2 months of age. These doses resulted in systemic exposures that were, respectively, 3, 20, and 60 times higher than the systemic exposure observed in humans following a subcutaneous dose of 20 mcg (based on AUC comparison). Teriparatide treatment resulted in a marked dose-related increase in the incidence of osteosarcoma, a rare malignant bone tumor, in both male and female rats. Osteosarcomas were observed at all doses and the incidence reached 40% to 50% in the high-dose groups. Teriparatide also caused a dose-related increase in osteoblastoma and osteoma in both sexes. No osteosarcomas, osteoblastomas or osteomas were observed in untreated control rats. The bone tumors in rats occurred in association with a large increase in bone mass and focal osteoblast hyperplasia.

The second 2-year study was carried out in order to determine the effect of treatment duration and animal age on the development of bone tumors. Female rats were treated for different periods between 2 and 26 months of age with subcutaneous doses of 5 and 30 mcg/kg (equivalent to 3 and 20 times the human exposure at the 20-mcg dose, based on AUC comparison). The study showed that the occurrence of osteosarcoma, osteoblastoma and osteoma was dependent upon dose and duration of exposure. Bone tumors were observed when immature 2-month old rats were treated with 30 mcg/kg/day for 24 months or with 5 or 30 mcg/kg/day for 6 months. Bone tumors were also observed when mature 6-month old rats were treated with 30 mcg/kg/day for 6 or 20 months. Tumors were not detected when mature 6-month old rats were treated with 5 mcg/kg/day for 6 or 20 months. The results did not demonstrate a difference in susceptibility to bone tumor formation, associated with teriparatide treatment, between mature and immature rats.

The relevance of these rat findings to humans is uncertain.

Mutagenesis

Teriparatide was not genotoxic in any of the following test systems: the Ames test for bacterial mutagenesis; the mouse lymphoma assay for mammalian cell mutation; the chromosomal aberration assay in Chinese hamster ovary cells, with and without metabolic activation; and the in vivo micronucleus test in mice.

Impairment of fertility

No effects on fertility were observed in male and female rats given subcutaneous teriparatide doses of 30, 100, or 300 mcg/kg/day prior to mating and in females continuing through gestation Day 6 (16 to 160 times the human dose of 20 mcg based on surface area, mcg/m^2).

Pregnancy

Pregnancy Category C — In pregnant rats given subcutaneous teriparatide doses up to 1000 mcg/kg/day, there were no findings. In pregnant mice given subcutaneous doses of 225 or 1000 mcg/kg/day (≥60 times the human dose based on surface area, mcg/m^2) from gestation Day 6 through 15, the fetuses showed an increased incidence of skeletal deviations or variations (interrupted rib, extra vertebra or rib).

Developmental effects in a perinatal/postnatal study in pregnant rats given subcutaneous doses of teriparatide from gestation Day 6 through postpartum Day 20 included mild growth retardation in female offspring at doses ≥225 mcg/kg/day (≥120 times the human dose based on surface area, mcg/m^2), and in male offspring at 1000 mcg/kg/day (540 times the human dose based on surface area, mcg/m^2). There was also reduced motor activity in both male and female offspring at 1000 mcg/kg/day. There were no developmental or reproductive effects in mice or rats at a dose of 30 mcg/kg (8 or 16 times the human dose based on surface area, mcg/m^2). The effect of teriparatide treatment on human fetal development has not been studied. FORTEO is not indicated for use in pregnancy.

Nursing Mothers

Because FORTEO is indicated for the treatment of osteoporosis in postmenopausal women, it should not be administered to women who are nursing their children. There have been no clinical studies to determine if teriparatide is secreted into breast milk.

Pediatric Use

The safety and efficacy of FORTEO have not been established in pediatric populations. FORTEO is not indicated for use in pediatric patients (*see* WARNINGS).

Geriatric Use

Of the patients receiving FORTEO in the osteoporosis trial of 1637 postmenopausal women, 75% were 65 years of age and over and 23% were 75 years of age and over. Of the patients receiving FORTEO in the osteoporosis trial of 437 men, 39% were 65 years of age and over and 13% were 75 years of age and over. No significant differences in bone response or adverse reactions were seen in geriatric patients receiving FORTEO as compared with younger patients. Nonetheless, as with many medications, elderly patients may have greater sensitivity to the adverse effects of FORTEO.

ADVERSE EVENTS

The safety of teriparatide has been evaluated in 24 clinical trials that enrolled over 2800 women and men. Four long-term Phase 3 clinical trials included 1 large placebo-controlled, double-blind, multinational trial with 1637 postmenopausal women; 1 placebo-controlled, double-blind, multinational trial with 437 men; and 2 active-controlled trials including 393 postmenopausal women. Teriparatide doses ranged from 5 to 100 mcg/day in short-term trials and 20 to 40 mcg/day in the other trials. A total of 1943 of the patients studied received teriparatide, including 815 patients at 20 mcg/day and 1107 patients at 40 mcg/day. In the clinical trials, a total of 1432 patients were treated with teriparatide for 3 months to 2 years, of whom 1137 were treated for greater than 1 year (500 at 20 mcg/day and 637 at 40 mcg/day). The maximum duration of treatment was 2 years. Adverse events associated with FORTEO usually were mild and generally did not require discontinuation of therapy.

In the two Phase 3 placebo-controlled clinical trials in men and postmenopausal women, early discontinuation due to adverse events occurred in 5.6% of patients assigned to placebo and 7.1% of patients assigned to FORTEO. Reported adverse events that appeared to be increased by FORTEO treatment were dizziness and leg cramps.

Table 5 lists adverse events that occurred in the two Phase 3 placebo-controlled clinical trials in men and postmenopausal women at a frequency ≥2.0% in the FORTEO groups and in more FORTEO-treated patients than in placebo-treated patients, without attribution of causality.

Table 5. Percentage of Patients with Adverse Events Reported by at Least 2% of FORTEO-Treated Patients and in More FORTEO-Treated Patients than Placebo-Treated Patients from the Two Principal Osteoporosis Trials in Women and Men
Adverse events are shown without attribution of causality.

	FORTEO N=691	Placebo N=691
Event Classification	(%)	(%)
Body as a Whole		
Pain	21.3	20.5
Headache	7.5	7.4
Asthenia	8.7	6.8
Neck pain	3.0	2.7
Cardiovascular		
Hypertension	7.1	6.8
Angina pectoris	2.5	1.6
Syncope	2.6	1.4
Digestive System		
Nausea	8.5	6.7
Constipation	5.4	4.5
Diarrhea	5.1	4.6
Dyspepsia	5.2	4.1
Vomiting	3.0	2.3
Gastrointestinal disorder	2.3	2.0
Tooth disorder	2.0	1.3
Musculoskeletal		
Arthralgia	10.1	8.4
Leg cramps	2.6	1.3
Nervous System		
Dizziness	8.0	5.4
Depression	4.1	2.7
Insomnia	4.3	3.6
Vertigo	3.8	2.7
Respiratory System		
Rhinitis	9.6	8.8
Cough increased	6.4	5.5
Pharyngitis	5.5	4.8
Dyspnea	3.6	2.6
Pneumonia	3.9	3.3
Skin and Appendages		
Rash	4.9	4.5
Sweating	2.2	1.7

Serum calcium — FORTEO transiently increases serum calcium, with the maximal effect observed at approximately 4 to 6 hours post-dose. Serum calcium measured at least 16 hours post-dose was not different from pretreatment levels. In clinical trials, the frequency of at least 1 episode of transient hypercalcemia in the 4 to 6 hours after FORTEO administration was

increased from 1.5% of women and none of the men treated with placebo to 11.1% of women and 6.0% of men treated with FORTEO. The number of patients treated with FORTEO whose transient hypercalcemia was verified on consecutive measurements was 3.0% of women and 1.3% of men.

Immunogenicity — In a large clinical trial, antibodies that cross-reacted with teriparatide were detected in 2.8% of women receiving FORTEO. Generally, antibodies were first detected following 12 months of treatment and diminished after withdrawal of therapy. There was no evidence of hypersensitivity reactions, allergic reactions, effects on serum calcium, or effects on BMD response.

OVERDOSAGE

Incidents of overdose in humans have not been reported in clinical trials. Teriparatide has been administered in single doses of up to 100 mcg and in repeated doses of up to 60 mcg/day for 6 weeks. The effects of overdose that might be expected include a delayed hypercalcemic effect and risk of orthostatic hypotension. Nausea, vomiting, dizziness, and headache might also occur.

In single-dose rodent studies using subcutaneous injection of teriparatide, no mortality was seen in rats given doses of 1000 mcg/kg (540 times the human dose based on surface area, mcg/m^2) or in mice given 10,000 mcg/kg (2700 times the human dose based on surface area, mcg/m^2).

Overdose management — There is no specific antidote for teriparatide. Treatment of suspected overdose should include discontinuation of FORTEO, monitoring of serum calcium and phosphorus, and implementation of appropriate supportive measures, such as hydration.

DOSAGE AND ADMINISTRATION

FORTEO should be administered as a subcutaneous injection into the thigh or abdominal wall. The recommended dosage is 20 mcg once a day.

FORTEO should be administered initially under circumstances in which the patient can sit or lie down if symptoms of orthostatic hypotension occur (*see* PRECAUTIONS, Information for the Patient).

FORTEO is a clear and colorless liquid. Do not use if solid particles appear or if the solution is cloudy or colored. The FORTEO pen should not be used past the stated expiration date.

No data are available on the safety or efficacy of intravenous or intramuscular injection of FORTEO.

The safety and efficacy of FORTEO have not been evaluated beyond 2 years of treatment. Consequently, use of the drug for more than 2 years is not recommended.

INSTRUCTIONS FOR PEN USE

Patients and caregivers who administer FORTEO should receive appropriate training and instruction on the proper use of the FORTEO pen from a qualified health professional. It is important to read, understand, and follow the instructions in the FORTEO pen User Manual for priming the pen and dosing. Failure to do so may result in inaccurate dosing. Each FORTEO pen can be used for up to 28 days after the first injection. After the 28-day use period, discard the FORTEO pen, even if it still contains some unused solution. Never share a FORTEO pen.

STORAGE

The FORTEO pen should be stored under refrigeration at 2° to 8°C (36° to 46°F) at all times. Recap the pen when not in use to protect the cartridge from physical damage and light. During the use period, time out of the refrigerator should be minimized; the dose may be delivered immediately following removal from the refrigerator.

Do not freeze. Do not use FORTEO if it has been frozen.

HOW SUPPLIED

The FORTEO pen is available in the following package size:
One 3 mL prefilled pen delivery device NDC 0002-8971-01 (MS8971)

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Manufactured by Lilly France S.A.S.
F-67640 Fegersheim, France
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Indianapolis, IN 46285, USA

www.lilly.com

Medication Guide

FORTEO™

Generic name: teriparatide (rDNA origin) injection

Read this information carefully before you start taking FORTEO (for-TAY-o) to learn about the benefits and risks of FORTEO. Before beginning therapy, read the FORTEO pen User Manual for information on how to use the pen to inject your medicine. Read the information you get with FORTEO each time you get a refill, in case something has changed. Talk with your health care provider if there is something you do not understand or if you want to learn more about FORTEO.

What is the most important information I should know about FORTEO?

As part of drug testing, teriparatide, the active ingredient in FORTEO, was given to rats for a significant part of their lifetime. **In these studies, teriparatide caused some rats to develop osteosarcoma, a bone cancer.** Osteosarcoma in humans is a serious but very rare cancer. Osteosarcoma occurs in about 4 out of every million older adults each year. **It is not known if humans treated with FORTEO also have a higher chance of getting osteosarcoma.**

FORTEO is approved for use in both men and postmenopausal (after the "change of life") women with osteoporosis who are at high risk for having broken bones (fractures) from osteoporosis.

Before starting treatment, talk with your doctor about the possible benefits and risks of FORTEO so you can decide if it is right for you.

What is osteoporosis?

Osteoporosis is a disease in which the bones become thin and weak, increasing the chance of having a broken bone. Osteoporosis usually causes no symptoms until a fracture happens. The most common fractures are in the spine (backbone). They can shorten height, even without causing pain. Over time, the spine can become curved or deformed and the body bent over. Fractures from osteoporosis can also happen in almost any bone in the body, for example, the wrist, rib, or hip. Once you have had a fracture, the chance for more fractures greatly increases.

The following risk factors increase your chance of getting fractures from osteoporosis:

- past broken bones from osteoporosis

- very low bone mineral density (BMD)
- frequent falls
- limited movement, such as using a wheelchair
- medical conditions likely to cause bone loss, such as some kinds of arthritis
- medicines that may cause bone loss, for example: seizure medicines (such as phenytoin), blood thinners (such as heparin), steroids (such as prednisone), high doses of vitamins A or D.

What is FORTEO?

FORTEO is a prescription medicine used to treat osteoporosis by forming new bone. FORTEO is the brand name for teriparatide, which is the same as the active part of a natural hormone called parathyroid hormone or "PTH." FORTEO forms new bone, increases bone mineral density and bone strength, and as a result, reduces the chance of getting a fracture. In a study of postmenopausal (after the "change of life") women with osteoporosis, FORTEO reduced the number of fractures of the spine and other bones. The effect on fractures has not been studied in men.

FORTEO is approved for use in both men and postmenopausal women with osteoporosis who are at high risk for having fractures. FORTEO can be used by people who have had a fracture related to osteoporosis, or who have multiple risk factors for fracture (See "What is osteoporosis?"), or who cannot use other osteoporosis treatments.

Who should not use FORTEO?

Do not use FORTEO if you:

- have Paget's disease of the bone
- have unexplained high levels of alkaline phosphatase in your blood, which means you might have Paget's disease. If you are not sure, ask your doctor.
- are a child or growing adult
- have ever been diagnosed with bone cancer or other cancers that have spread (metastasized) to your bones
- have had radiation therapy involving your bones
- have certain bone diseases. If you have a bone disease, tell your doctor.
- have too much calcium in your blood (hypercalcemia)
- are pregnant or nursing
- have had an allergic reaction to FORTEO or one of its ingredients (See the ingredients section at the end of this Medication Guide)
- have trouble injecting yourself and do not have someone who can help you.

FORTEO should not be used to prevent osteoporosis or to treat patients who are not considered to be at high risk for fracture.

Tell your health care provider and pharmacist about all the medicines you are taking when you start taking FORTEO, and if you start taking a new medicine after you start FORTEO treatment. Tell them about all medicines you get with prescriptions and without prescriptions, as well as herbal or natural remedies. Your doctor and pharmacist need this information to help keep you from taking a combination of products that may harm you.

How should I take FORTEO?

- Take FORTEO once a day for as long as your doctor prescribes it for you. Use of FORTEO for more than 2 years is not recommended. Your health care professional (doctor, nurse, or pharmacist) should teach you how to use the FORTEO pen (prefilled delivery device). (See the User Manual for written instructions on how to use the FORTEO pen.)
- Some patients get dizzy or get a fast heartbeat after the first few doses. For the first few doses, inject FORTEO where you can sit or lie down right away if you get dizzy.
- Inject FORTEO once each day in your thigh or abdomen (lower stomach area).
- You can take FORTEO with or without food or drink.
- You can take FORTEO at any time of the day. To help you remember to take FORTEO, take it at about the same time each day.
- Do not use FORTEO if it has solid particles in it, or if it is cloudy or colored. It should be clear and colorless.
- Do not use FORTEO after the expiration date printed on the pen and pen packaging.
- Throw away any FORTEO pen that you started using more than 28 days earlier, even if it still has medicine in it (See the User Manual).
- Inject FORTEO shortly after you take the pen out of the refrigerator. Recap the pen and put it back into the refrigerator right after use (See the User Manual).
- If you forget or are unable to take FORTEO at your usual time, take it as soon as possible on that day. Do not take more than one injection in the same day.
- Talk with your health care provider about other ways you can help your osteoporosis, such as exercise, diet, supplements, and reducing or stopping your use of tobacco and alcohol. If your health care provider recommends calcium and vitamin D supplements, you can take them at the same time as FORTEO.

What are the possible side effects of FORTEO?

Most side effects are mild and include dizziness and leg cramps. If you become lightheaded or have fast heartbeats after your injection, sit or lie down until you feel better. If you do not feel better, call your health care provider before continuing treatment.

Contact your health care provider if you have continuing nausea, vomiting, constipation, low energy, or muscle weakness. These may be signs there is too much calcium in your blood.

These are not all the possible side effects of FORTEO. For more information, ask your health care provider or pharmacist.

Your health care provider may take samples of blood and urine during treatment to check your response to FORTEO. Also, your health care provider may ask you to have follow-up tests of bone mineral density.

How should I store FORTEO?

- Keep your FORTEO pen in the refrigerator at 36° to 46°F (2° to 8°C).
- Do not freeze the pen. Do not use FORTEO if it has been frozen.
- You can use your FORTEO pen for up to 28 days after the first injection from the pen.
- Throw away the pen properly (See the User Manual) after 28 days of use, even if it is not completely empty.
- Recap the pen after each use (See the User Manual) to protect from physical damage.

General information about using FORTEO safely and effectively

Medicines are sometimes prescribed for conditions that are not mentioned in Medication Guides. Do not use FORTEO for a condition for which it was not prescribed. Do not give FORTEO to other people, even if they have the same condition you have.

This Medication Guide summarizes the most important information about FORTEO. If you would like more information, talk with your doctor, nurse, or pharmacist. You can ask your pharmacist or health care provider for information about FORTEO that is written for health care professionals. You can also call Lilly toll free at 1-866-4FORTEO (1-866-436-7836).

Ingredients

In addition to the active ingredient teriparatide, inactive ingredients are glacial acetic acid, sodium acetate (anhydrous), mannitol, Metacresol, and Water for Injection. In addition, hydrochloric acid solution 10% and/or sodium hydroxide solution 10% may have been added to adjust product pH.

This Medication Guide has been approved by the US Food and Drug Administration.

Literature issued November 2002

Manufactured by Lilly France S.A.S.
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SA483

In Vitro Inhibition of Bone Resorption by Human PTH(7-84).

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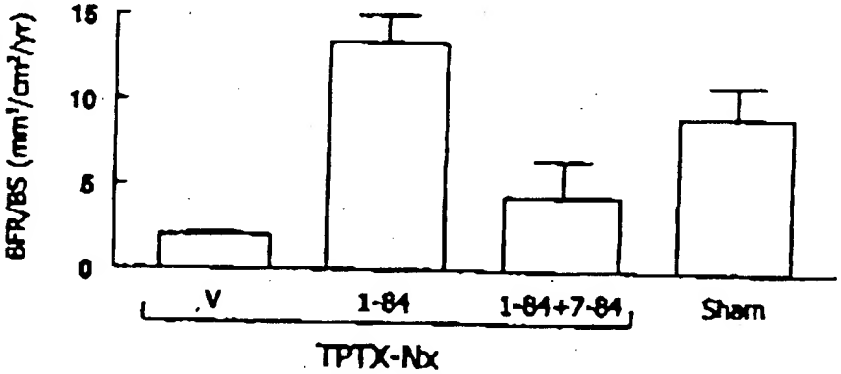
Intact PTH from different mammalian species comprises 84 amino acids and shows high sequence conservation within both its amino (N)-terminal and carboxyl (C)-terminal regions. The N-terminal 34 amino acids are sufficient for classical actions of intact PTH in the regulation of mineral ion homeostasis and bone metabolism, which are known to be mediated through activation of the type 1 PTH/PTHrP receptor (PTH1R). C-terminal PTH (CPTH) fragments, analogous to those known to be released into blood during peripheral proteolysis of intact PTH or via direct parathyroid secretion, exert biologic effects in osteoblastic and chondrocytic cells and bind specifically to a putative CPTH receptor in such cells. Recently large CPTH fragments, minimally truncated at their N-termini and cross-reactive with most commercially available intact PTH immunoassays, were detected in normal plasma and, at higher levels, in plasma of patients with advanced renal failure. These fragments exhibit chromatographic properties similar to synthetic PTH(7-84). Moreover, hPTH(7-84) recently was shown to inhibit the calcemic actions of PTH(1-84) and PTH(1-34) in parathyroidectomized rats.To determine if hPTH(7-84) might antagonize the calcemic response to PTH(1-84) in vivo via direct effects on bone, we tested hPTH(7-84) for inhibition of in vitro assays of osteoclast formation (murine marrow cultures) and bone resorption (release of 45Ca from intact murine calvarial bone). Addition of hPTH(7-84) alone (300nM) reduced basal 45Ca release by approximately 50% (control: 17.8±5.7%; hPTH(7-84): 9.6± 1.9% ; $p < 0.001$), an effect comparable to that of salmon calcitonin. HPTH(7-84) also inhibited agonist-induced bone resorption caused by a variety of agents, including intact PTH(1-84), PTH(1-34), 1,25(OH)2D3 (1,25D), prostaglandin E2 and interleukin-11. In murine marrow cultures, 1,25D (10 nM) stimulated the formation of TRAP positive cells by 13.9 fold; in the presence of 300nM PTH(7-84), this response was reduced by 65%. It is unlikely that this inhibitory effect of PTH(7-84) was due to an antagonistic effect at the PTH1R, as neither hPTH(3-34) nor [L11,DW12]PTHrP(7-36), both potent PTH1R antagonists in these systems, inhibited bone resorption induced by PTH(1-34) or 1,25D-induced osteoclast formation. We conclude that hPTH(7-84), acting via receptors (probably CPTH receptors) independent of the PTH1R, can exert a generalized antiresorptive effect that may involve both inhibition of osteoclast recruitment and reduction in formation or activation of mature osteoclasts.

A3995 F54 (FC) SU1-0859 (PS)

The Effects of PTH-(1-84) on Bone Turnover Are Antagonized by PTH-(7-84) in Thyroparathyroidectomized and Nephrectomized Rats.

Marie-Claude Faugere, Moises C. Langub, Hartmut H. Malluche. *Nephrology, Bone & Mineral Metabolism, University of Kentucky, Lexington, KY.*

PTH-(7-84)antagonizes the effects of PTH-(1-84) on rise in serum calcium in thyroparathyroidectomized (TPTX) rats. To determine whether PTH-(7-84)also can antagonize the effects of PTH-(1-84) on bone turnover, 18 rats were TPTX and nephrectomized (Nx) and 6 rats were sham-operated (Sham). TPTX-Nx rats were divided in 3 groups (n = 6 each) and allocated to receive vehicle (Veh), hPTH-(1-84) at a dose of 216 ng/kg.hr alone or in combination with hPTH-(7-84) at a dose of 4,000 ng/kg.hr. Sham rats received Veh. Doses were administered s.c. via Alzet minipumps for 2 weeks. At euthanasia blood was collected and femurs excised for histomorphometry after calcein labeling. All TPTX-Nx rats had higher serum creatinine compared to sham rats (0.89 ± 0.04 vs. 0.53 ± 0.03 mg/dl, $p<0.05$). Serum calcium levels were lower in TPTX-Nx/V than in Sham (7.7 ± 0.26 vs. 10.4 ± 0.13 mg/dl, $p<0.05$). Hypocalcemia was corrected in rats given PTH-(1-84) alone (10.2 ± 0.33 mg/dl) whereas addition of PTH-(7-84) blunted this response (8.3 ± 0.27 mg/dl). Parameters of bone turnover were strongly suppressed in TPTX-Nx/Veh (Fig.). PTH-(1-84) restored bone turnover to sham levels whereas PTH-(7-84) blunted this response (Fig.).



The results indicate that PTH-(7-84) is not only antagonistic to the calcemic effects of PTH-(1-84), but also to the stimulatory effects of PTH-(1-84) on bone turnover. These data ascribe a potential role to PTH-(7-84) in the pathogenesis of adynamic bone disease.

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Improved assessment of bone turnover by the PTH-(1-84)/large C-PTH fragments ratio in ESRD patients

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Improved assessment of bone turnover by the PTH-(1-84)/large C-PTH fragments ratio in ESRD patients.

Background. The "intact" parathyroid hormone (PTH) assay recognizes PTH-(1-84) as well as amino terminally truncated PTH fragments, that is, large carboxyterminal PTH fragments (C-PTH fragments). The present study investigated whether the use of the plasma PTH-(1-84)/C-PTH fragment ratio enhances the noninvasive assessment of bone turnover in patients on dialysis.

Methods. Bone biopsies and blood samples for determinations of routine indices of bone turnover and PTH peptides were obtained in 51 adult patients on dialysis not treated with drugs affecting bone such as vitamin D or corticosteroids. Blood levels of large C-PTH fragments were calculated by subtracting PTH-(1-84) from "intact" PTH. Patients were classified according to their levels of bone turnover based on histomorphometrically obtained results of activation frequency. Prediction of bone turnover by the various blood indices was done by using proper statistical methods. In addition, hypercalcemia was induced by calcium gluconate infusion in a subset of patients, and levels of PTH-(1-84), "intact" PTH, and PTH-(1-84)/C-PTH fragment ratio were determined.

Results. The PTH-(1-84)/C-PTH fragment ratio was the best predictor of bone turnover. A ratio >1 predicted high or normal bone turnover (sensitivity 100%), whereas a ratio <1 indicated a high probability (sensitivity 87.5%) of low bone turnover. Calcium infusion resulted in decrease in PTH-(1-84)/C-PTH fragment ratio.

Conclusions. The PTH-(1-84)/C-PTH fragment ratio predicts bone turnover with acceptable precision for biological measurements. Moreover, a change in serum calcium levels is one of the regulators of the relative amount of circulating PTH-(1-84) and its large C-PTH fragments.

Virtually all patients with end-stage renal disease (ESRD) develop mineral and bone abnormalities secondary to the loss of kidney function. These abnormalities have a substantial impact on the morbidity and mortality of these patients. Despite an initially common pathogenetic pathway, there are no uniform histopathologic changes among the patients. Patients on dialysis present either with various degrees of secondary hyperparathyroidism, with or without mineralization defect, resulting in high or normal bone turnover or with adynamic bone characterized by low bone turnover. Distinction between high or normal versus low bone turnover is essential because these entities require divergent therapeutic approaches, that is, indication for vitamin D and level of dose aggressiveness, use of calcium- versus non–calcium-containing phosphate binders, and choice of calcium dialysate concentration. Currently, histologic analysis of bone after tetracycline labeling remains the gold standard for assessment of bone turnover. However, this technique is not always available. Physicians generally rely on noninvasive methods, particularly on blood concentrations of intact parathyroid hormone (PTH). However, the level of blood PTH that predict low or high-normal bone turnover has not been clearly established.

In the early 1990s, it was advocated that adequate control of secondary hyperparathyroidism, that is, normal bone turnover, is achieved when blood levels of intact PTH are between one and four times the upper limit of normal range [1–5]. However, we and others found low bone turnover on bone histology in patients on dialysis with intact PTH levels of approximately 10 times the upper limit of normal [6, 7]. Because various degrees of superimposed bone aluminum accumulation may have contributed to these discrepancies between studies, we recently revisited the value of intact PTH levels for assessment of bone turnover in 157 patients on dialysis (abstract; Monier-Faugere et al, *J Am Soc Nephrol* 11:554A, 2000) [8]. These patients were given exclusively calcium salts for phosphate binding and did not show any histologically proven aluminum bone accumulation. We found that 89.2% of patients with intact

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PTH levels ≤100 pg/mL had low bone turnover, whereas 89.4% patients with levels ≥500 pg/mL had high or normal bone turnover. However, in the majority of the patients, that is, in patients with intact PTH between 100 and 500 pg/mL, low bone turnover was observed in 59% and high or normal in the remaining 41% of the patients.

The characteristics of the commercially available kits for determinations of intact PTH may account, at least in part, for the relative poor predictability of intact PTH in assessing bone turnover. Indeed, it has been demonstrated that two immunoreactive components can be detected with available assays for intact PTH [9]. One component comigrates with synthetic human PTH-(1-84), whereas the other non-(1-84) component is more hydrophilic and accumulates in renal failure, accounting for 40 to 60% of the total immunoreactivity in ESRD patients [9]. Subsequently, it was shown that the non-(1-84) PTH molecule comigrates closely with the synthetic human 7-84 PTH fragment [10]. Recently, a novel immunoradiometric assay was developed that exclusively detects the full-length 1-84 but not PTH fragments [11, 12]. Combining the results employing this new PTH-(1-84) assay with high-performance liquid chromatography (HPLC) profiles, it was demonstrated that the non-(1-84) PTH consists of amino terminally truncated PTH fragments, that is, large carboxyterminal PTH fragments (C-PTH fragments) [12], likely PTH-(7-84).

The C-PTH fragments have long been considered inactive peptides. However, it has been shown that they regulate alkaline phosphatase, osteocalcin, collagen α1(I), and insulin growth factor binding protein-5 in rat and human osteoblast-like cells [13–16]. They also stimulate the proliferation and activity of osteoclasts [17] and stimulate activity of hypertrophic chondrocytes [18, 19]. Also, differences in the action of PTH-(1-84) and PTH-(1-34) on various cells have been noted [20–23], pointing to a role of the C-PTH fragments in the activity of PTH. In thyroparathyroidectomized rats, the administration of human PTH-(7-84) alone or in a mixture of other C-PTH fragments such as PTH-(39-84) and PTH-(53-84) antagonized the calcemic response elicited by PTH-(1-84) [24, 25]. Moreover, several binding studies have demonstrated the presence of C-PTH receptors in kidney and bone cells [13, 25–31], especially in osteocytes [31].

Because the large C-PTH fragments contain portions of PTH essential for binding to either the PTH-1 receptor [24, 32–36] or the C-PTH receptor [13, 25–31], the large C-PTH fragments have the potential to antagonize PTH-(1-84) action on bone.

In the present study, we assume that endogenous C-PTH fragments in blood of patients on dialysis are antagonists of the effects of PTH-(1-84) on bone turnover. We hypothesize that the C-PTH fragments act as a negative biologic regulator of the stimulatory effects of PTH-(1-84) on bone turnover, and thus, the PTH-(1-84)/C-PTH fragment ratio has more predictive power in distinguishing low and high bone turnover than the use of PTH-(1-84) alone.

The present study was undertaken (*1*) to test the previously mentioned hypothesis and (*2*) to determine the effects of hypercalcemia on the PTH-(1-84)/C-PTH.

METHODS

Patients

Patients were recruited prospectively from local dialysis clinics during the years 1999 to 2000. The inclusion criteria were age above 18 years and willingness to undergo a bone biopsy and blood drawing. The exclusion criteria were a history of past or present treatment with aluminum phosphate binders, treatment with calcitriol or medications known to affect bone metabolism (diphenylhydantoin, glucocorticoids, cyclosporine) during the last six months, systemic illnesses or organ diseases other than diabetes that may affect bone metabolism (that is, gastrointestinal diseases, liver disease, malignancies, tuberculosis, acquired immunodeficiency syndrome, chronic alcoholism, drug addiction, failed transplant or parathyroidectomy within the last six months, participation in other studies), or tetracycline allergy.

One hundred thirty-five patients were screened. Sixty-five were eligible and 51 agreed to participate in the study; informed consents were signed. There were 29 men and 22 women with a mean age of 47 ± 3 and 43 ± 3 years, respectively. Thirty-two patients were on hemodialysis (HD), and 19 patients were on chronic ambulatory peritoneal dialysis (CAPD). The mean duration on dialysis treatment was 25.6 ± 3.0 months (2 months to 7 years). Underlying kidney diseases were hypertensive nephropathy ($N = 18$), diabetes mellitus ($N = 12$), glomerulonephritis ($N = 8$), interstitial nephritis ($N = 7$), miscellaneous nephropathies ($N = 5$), and unknown origin ($N = 1$). HD patients were dialyzed three times a week for three or four hours. Patients on CAPD underwent four to five 2 to 2.5 L exchanges per day. Calcium dialysate was 2.5 mEq/L. Only routine dialysis support medications were given, including calcium salts for phosphate binding.

Protocol

After signing the consent form, patients were scheduled to undergo an iliac crest bone biopsy. Before bone biopsy, patients received double tetracycline labeling of bone as previously described [37]. During the week before bone biopsies, on days when bone labels were not administered, blood samples were obtained after a 12-hour fast for measurement of circulating calcium, phosphorus, intact PTH, PTH-(1-84), bone-specific alkaline phosphatase (BSAP), and osteocalcin levels. In a subset of six patients, two to four weeks after the bone biopsy,

in vivo dynamic tests of parathyroid gland function, that is, effects of hypercalcemia on PTH peptides, were performed.

Bone biopsy, mineralized bone histology, and bone histomorphometry

Anterior iliac crest bone biopsies were done under local anesthesia and conscious sedation. Bone samples were obtained with the one-step electrical drill technique (Straumann Medical, Waldenburg, Switzerland) as previously described [37].

Bone samples were processed undecalcified as previously described [38]. Sections were stained with the modified Masson-Goldner trichrome stain [39], the aurin tricarboxylic acid stain [40], and solochrome azurin [41]. Unstained sections were prepared for phase contrast and fluorescent light microscopy.

Histomorphometric analysis of bone was done at standardized sites in cancellous bone using the semiautomatic method (Osteoplan II; Kontron, Munich, Germany) at a magnification of ×200 [42, 43]. Activation frequency a parameter, which includes both bone formation and resorption, was measured for determination of bone turnover. In the present study, there was a strong relationship between activation frequency and bone formation rate/bone surface (BFR/BS; $r = 0.96$, $P < 0.001$).

Induction of hypercalcemia

Serum calcium was raised during a two-hour infusion of 10% calcium gluconate (9.3 mg Ca^{2+}/mL) according to the method of Ramirez et al [44]. The initial dose of calcium was 2 mg/kg · hour and was increased thereafter by 1 mg/kg · hour every 20 minutes. Blood was collected 30, 15, and 0 minutes before infusion and every 10 minutes thereafter for determinations of serum ionized calcium, intact PTH levels, and plasma PTH-(1-84) concentrations.

Biochemistry

Serum calcium and phosphorus were measured by standard laboratory techniques. Ionized calcium was determined using a Radiometer Copenhagen (Westlake, OH, USA). Plasma intact PTH levels were determined with the IRMA assay for intact PTH (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The reference range is 15 to 65 pg/mL. The intra-assay and interassay coefficients of variation were 3.4 and 5.6%, respectively. Plasma PTH-(1-84) was determined with the IRMA assay using a radiolabeled detection antibody specific for the first amino acid from the N-terminal site (Whole PTH™; Scantibodies, Inc., Santee, CA, USA) [11, 12]. The reference range of the assay is 7 to 36 pg/mL based on over 120 normal controls [12]. Intra-assay and interassay coefficients of variation were <5 and <7%, respectively. Plasma concentrations of the C-PTH fragments were determined by subtracting the PTH-(1-84) circulating levels from the results of the intact PTH. The PTH-(1-84)/C-PTH fragments ratio was then calculated. Serum osteocalcin levels were measured with the IRMA Human Osteocalcin 100T kit (Nichols Institute Diagnostics). The reference range was 2.0 to 10.0 ng/mL. The intra-assay and interassay variations were 5.3 and 5.7%, respectively. Serum BSAP levels were determined using an IRMA assay (Tandem-R Ostase; Hybritech, San Diego, CA, USA). Reference ranges are 3.9 to 20.9 μg/L for men and 2.9 to 20.1 μg/L for women. The intra-assay and interassay coefficients of variation were 6.7 and 8.1%, respectively.

Statistical analysis

Patients were classified as having high or normal bone turnover if activation frequency was above the lower limit of reference range obtained from bone samples of normal age- and gender-matched volunteers processed and analyzed in our laboratory (>0.42 $year^{-1}$). In the same manner, patients were classified as having low bone turnover if activation frequency was below 0.42 $year^{-1}$.

Results are expressed as mean ± SEM. All statistical tests were two sided. An assigned significance level of 0.05 was used. Normality of distribution was assessed by the Lilliefors test, and homogeneity of variance was tested with the Levene test. Adequate transformations of the data were done for serum calcium (reciprocal) and plasma intact PTH (square root) [45]. Comparisons of continuous values between bone turnover groups were performed by the Student t test. The chi-square test was used for categorical variables. Comparisons between results of CAPD and HD patients were done using the Student t test. Pearson's coefficients of correlation were obtained between activation frequency and demographic and biochemical variables. Logistic regression was performed to detect predictive factors of activation frequency. Computations and analyses were performed using SPSS 7.5 software package for Windows (SPSS Inc., Chicago, IL, USA).

Post-test probability parameters (sensitivity, specificity, predictive value positive and negative), receiver-operator characteristics (ROC) curves, and areas under the curves were obtained using Analyse-It software for Microsoft Excel, version 1.5 (Analyse-It Software, LTD, Leeds, UK). ROC curves are a plot of the true positive rate (sensitivity) against false positive rate (1 − specificity), and the area under the curve is a measure of test accuracy. Comparisons between the areas under the curves were done with the same software using the Hanley and McNeil methods [46]. In addition, Youden indices (sensitivity + specificity − 1) were calculated for determination of the best cut-off level of a biochemical parameter, which provides the best threshold for diagnosis of a disease, in the present study low bone turnover [47].

Table 1. Demographics, clinical, and biochemical characteristics of 51 chronically dialyzed patients according to levels of bone turnover

	Low bone turnover	High or normal bone turnover
Number of patients	28	23
Age *years*	50 ± 3	39 ± 3*
Patients on HD/CAPD	16/12	16/7
Male/female	18/10	11/12
Diabetic patients	8/20	4/18
Duration on dialysis *months*	26 ± 4	25 ± 4
Serum calcium *mg/dL*	9.3 ± 0.2	9.0 ± 0.2
Ionized calcium *mEq/L*	4.35 ± 0.11	4.70 ± 0.08
Serum phosphorus *mg/dL*	6.1 ± 0.4	7.1 ± 0.5
Serum bone-specific alkaline phosphatase *μg/L*	19.8 ± 3.45	35.1 ± 4.39*
Serum osteocalcin *ng/mL*	21.5 ± 4.8	34.5 ± 5.1

Abbreviations are: HD, hemodialysis; CAPD, chronic ambulatory peritoneal dialysis.
* Different from low bone turnover, $P < 0.01$, student t test

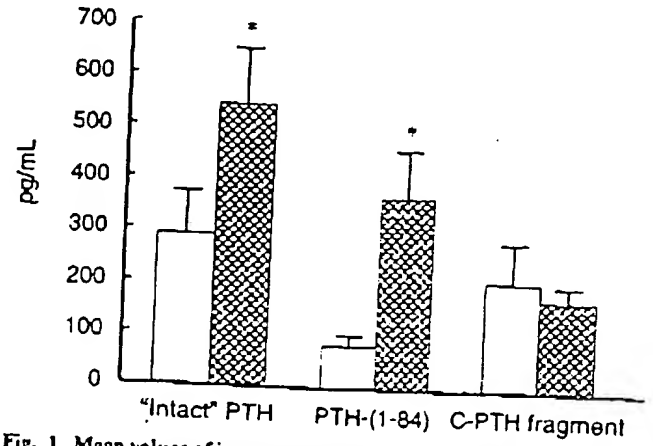

Fig. 1. Mean values of intact parathyroid hormone (PTH), parathyroid hormone (1-84) [PTH-(1-84)], and large carboxyterminal-PTH (C-PTH) fragments (C-PTH fragments) in 51 patients on chronic maintenance dialysis with low bone turnover (□) and high or normal bone turnover (▨). The asterisk indicates significant differences between high or normal and low bone turnover ($P < 0.01$).

RESULTS

Bone turnover in the studied patients

More than half of the patients exhibited low bone turnover, whereas high or normal bone turnover was found in approximately 45% (Table 1). None of the bone samples exhibited stainable aluminum deposits. Patients with low bone turnover were older than those with high or normal bone turnover (Table 1). Low bone turnover also was found more frequently in diabetics and patients on CAPD; however, this did not reach statistical significance (Table 1). There were no differences in gender distribution, age, number of diabetic patients, duration on dialysis, or biochemical and hormonal parameters between patients on HD and CAPD.

Biochemical and hormonal parameters

Serum calcium and phosphorus levels were similar in patients with various levels of bone turnover (Table 1). Serum BSAP levels were significantly higher in patients with high or normal than low bone turnover (Table 1). Higher serum osteocalcin levels were associated with high or normal bone turnover; however, this did not reach statistical significance (Table 1). Both plasma intact and PTH-(1-84) were significantly higher in patients with high or normal than low bone turnover, whereas the calculated C-PTH fragments were similar (Fig. 1). Patients with low bone turnover had significantly more C-PTH fragments than PTH-(1-84) ($P < 0.001$), whereas patients with high or normal bone turnover had significantly more PTH-(1-84) than C-PTH fragments ($P < 0.001$; Fig. 1), and the PTH-(1-84)/C-PTH fragment ratio was significantly higher in patients with high or normal (range of 0.47 to 14.2) than low bone turnover (range of 0.01 to 0.99; Fig. 2).

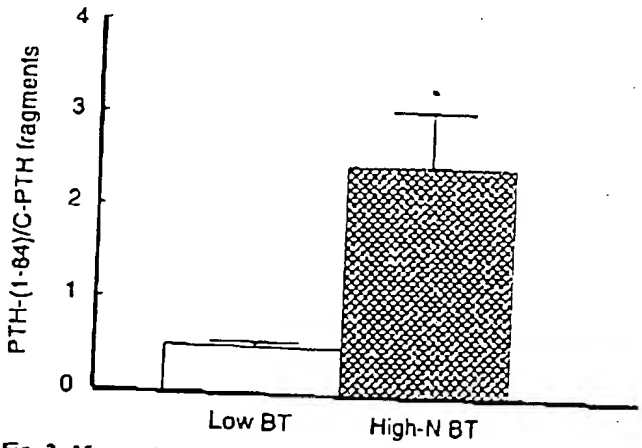

Fig. 2. Mean value of the PTH-(1-84)/C-PTH fragments ratio in 51 patients on chronic maintenance dialysis with low bone turnover (low BT) and high or normal bone turnover (high-N BT). The asterisk indicates significant difference between high or normal and low bone turnover ($P < 0.01$).

Relationship between blood indices and bone turnover

Relationships between plasma intact PTH, PTH-(1-84), PTH-(1-84)/C-PTH fragments, and BSAP and activation frequency (bone turnover) are shown in Figure 3. PTH-(1-84) and PTH-(1-84)/C-PTH fragment ratio correlated best with activation frequency ($r = 0.73$ and 0.68, $P < 0.01$, respectively), whereas intact PTH, osteocalcin, and BSAP had somewhat lower coefficients of correlation (0.51, 0.48, and 0.42, $P < 0.01$, respectively). Serum total and ionized calcium and phosphorus did not correlate with any of the PTH peptides, BSAP, or osteocalcin. All data obtained using BFR/BS as end point yielded results comparable to those obtained with activation frequency.

The PTH-(1-84) levels showed the best relationship

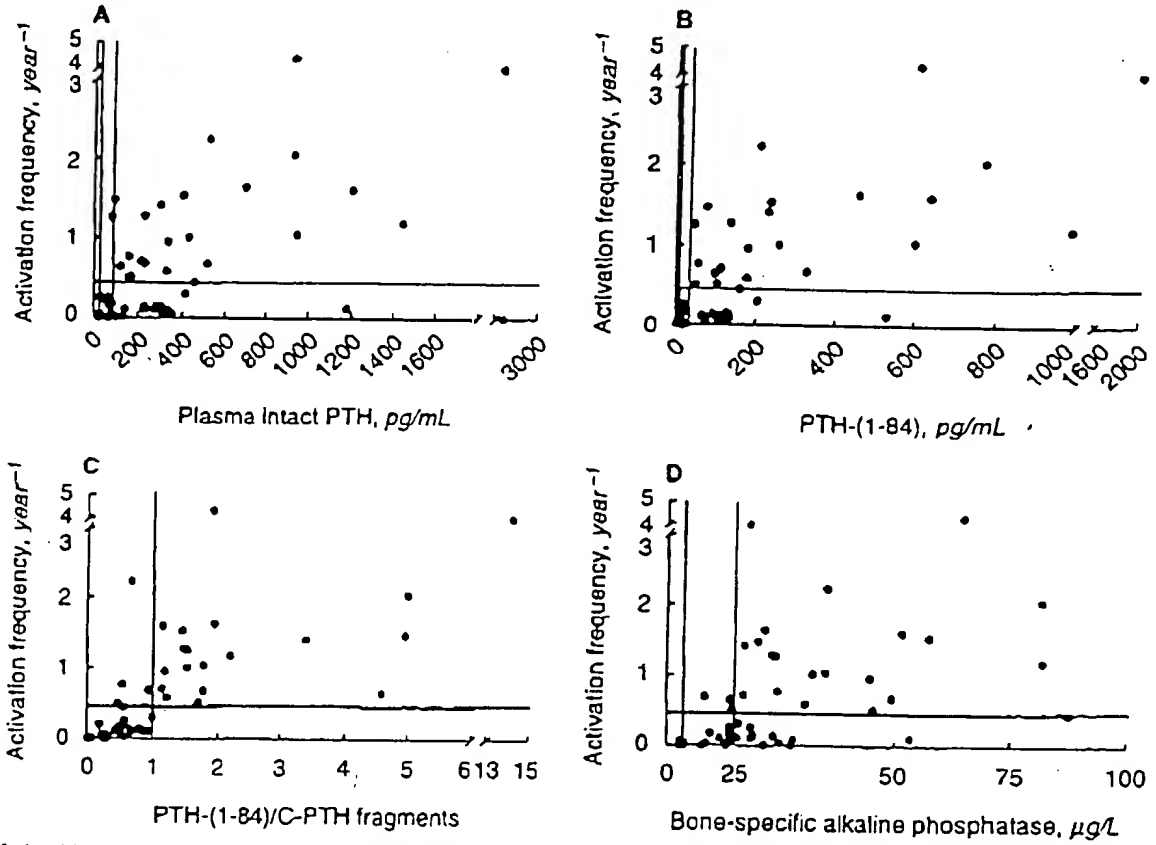

Fig. 3. Relationships between intact PTH, PTH-(1-84), PTH-(1-84)/C-PTH fragment ratio, and bone-specific alkaline phosphatase and bone turnover (activation frequency) in 51 patients on chronic maintenance dialysis.

with bone turnover; however, correlations are driven, at least in part, by the extreme values, whereas the predictive value of a test is determined by the distribution of the intermediate values (slope).

Determination of the best predictor of bone turnover

To determine the factor(s) that predicts bone turnover, we performed logistic regression and post-test probability.

Logistic regression pointed to the PTH-(1-84)/C-PTH fragments ratio as the only parameter predicting bone turnover (activation frequency, Ac.f, $P < 0.001$) with an overall predictability of 88.2%.

$$Ac.f = \frac{1}{1 + e^{-(-5.2+5.5\ \text{ratio})}}$$

Post-test probability parameters for the PTH-(1-84)/C-PTH fragment ratio to predict low bone turnover are shown in Table 2. Their composite results, that is, the ROC curves for PTH-(1-84)/C-PTH fragments ratio as well as for PTH-(1-84), intact PTH, BSAP, and osteocalcin are shown in Figure 4. The area under the curve for the PTH-(1-84)/C-PTH fragment ratio was significantly greater than those of PTH-(1-84) ($P < 0.05$), BSAP ($P < 0.05$), intact PTH ($P < 0.01$), and osteocalcin ($P < 0.01$; Fig. 5). The area under the curve was also significantly greater for PTH-(1-84) than for intact PTH and osteocalcin ($P < 0.05$; Fig. 5). There was no difference between BSAP, osteocalcin, and intact PTH (Fig. 5).

Determination of the best cut-off point

To determine the best cut-off level of the PTH-(1-84)/C-PTH fragment ratio, the Youden indices for the PTH-(1-84)/C-PTH fragment ratio were calculated for every level of the ratio. Given that a perfect cut-off would have a Youden index of 1, the highest Youden index (0.826) was found for a value of PTH-(1-84)/C-PTH fragment ratio of 1 (Fig. 6).

To test how this cut-off point discriminates between levels of bone turnover, we determined the number of patients correctly diagnosed with a PTH-(1-84)/C-PTH fragment ratio above or below 1 (Fig. 3). All 19 patients with a PTH-(1-84)/C-PTH fragment ratio above 1 had normal or high bone turnover. The 32 patients with a ratio less than 1 exhibited low bone turnover except in 4 patients who had either normal ($N = 2$) or high bone turnover ($N = 2$). No other parameters, alone or in

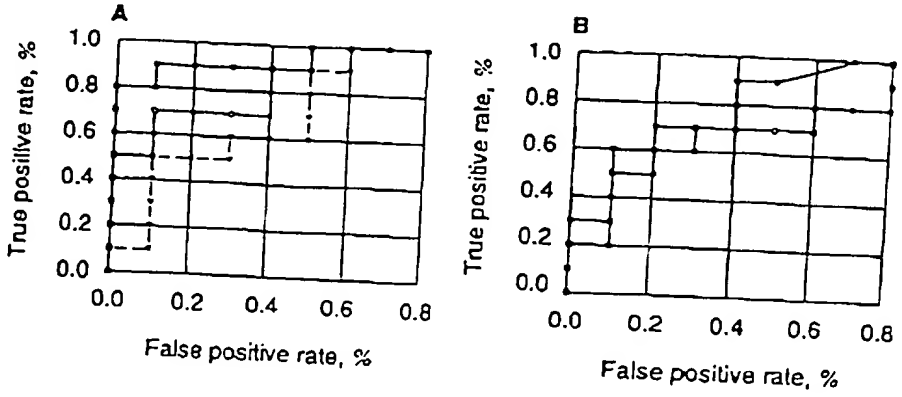

Fig. 4. Receiver-operator characteristics (ROC) curves for the prediction of bone turnover in chronically dialyzed patients. (A) Symbols are: plasma PTH-(1-84)/C-PTH fragment ratio (■), plasma PTH-(1-84) (○), and plasma intact PTH (▼). (B) Symbols are: serum bone-specific alkaline phosphatase (■) and serum osteocalcin (□).

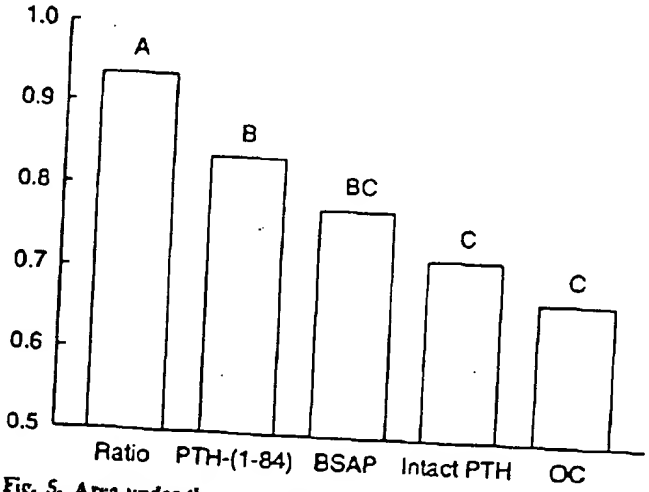

Fig. 5. Area under the curve (AUC) of the receiver-operator characteristics (ROC) curves for PTH-(1-84)/C-PTH fragments ratio, PTH-(1-84), bone-specific alkaline phosphatase (BSAP), intact PTH, and osteocalcin (OC). Values with the same letter are not significantly different.

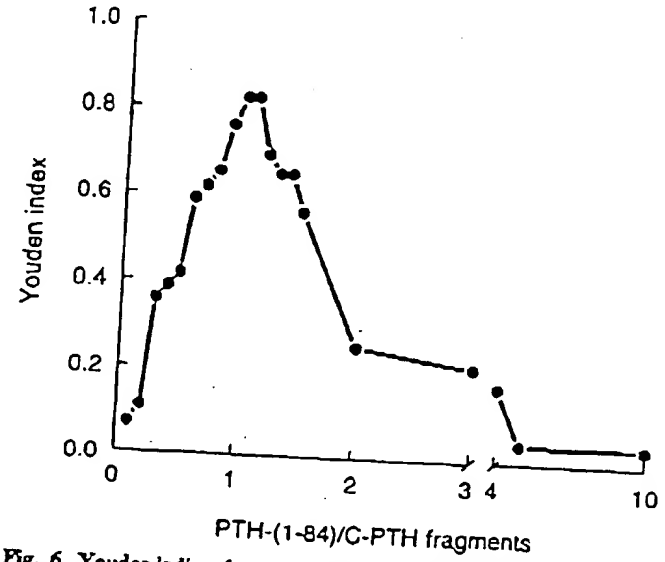

Fig. 6. Youden indices for determination of the optimal cut-off level of the PTH-(1-84)/C-PTH fragments ratio for prediction of bone turnover.

Table 2. Post-test probability of PTH-(1-84)/C-PTH fragments PTH ratio for prediction of low bone turnover

PTH-(1-84)/C-PTH fragments	Sens	Spec	PVP	PVN
		%		
<0.3	35.7	100	100	56.1
<0.4	39.2	100	100	57.5
<0.5	46.4	95.6	92.8	59.5
<0.6	67.8	91.3	90.5	70.0
<0.7	75.0	86.9	87.7	74.1
<0.8	78.6	86.9	88.0	76.9
<0.9	89.3	86.9	89.3	86.9
<1.0	100	82.6	87.5	100
<1.1	100	82.6	87.5	100
<1.2	100	69.5	80.0	100

Abbreviations are: Sens, sensitivity; Spec, specificity; PVP, predictive value for positive results; PVN, predictive value for negative results.

combination, including serum calcium, identified these four patients. When patients with blood intact PTH levels between 100 and 500 pg/mL were analyzed separately, a PTH-(1-84)/C-PTH fragment ratio >1 was also 100% diagnostic for high or normal bone turnover, whereas a ratio <1 was 82% diagnostic for low bone turnover.

Effects of hypercalcemia on the PTH-(1-84)/C-PTH fragments ratio

Induction of hypercalcemia by infusion of calcium gluconate in six patients resulted in the progressive decrease in intact PTH, PTH-(1-84), as well as the PTH-(1-84)/C-PTH fragment ratio with time (Fig. 7). Moreover, there was an inverse relationship between serum-ionized calcium levels and the PTH-(1-84)/C-PTH fragment ratio in individual patients (Fig. 8).

DISCUSSION

The novel information provided by the present study is the superiority of the PTH-(1-84)/C-PTH fragment ratio in predicting bone turnover compared with all other biochemical parameters, alone or in combination, thus supporting our central hypothesis. Bone turnover is reflected by the balance between the relative amount of circulating PTH-(1-84) and large C-PTH fragments. Predominance of circulating active PTH-(1-84) over the

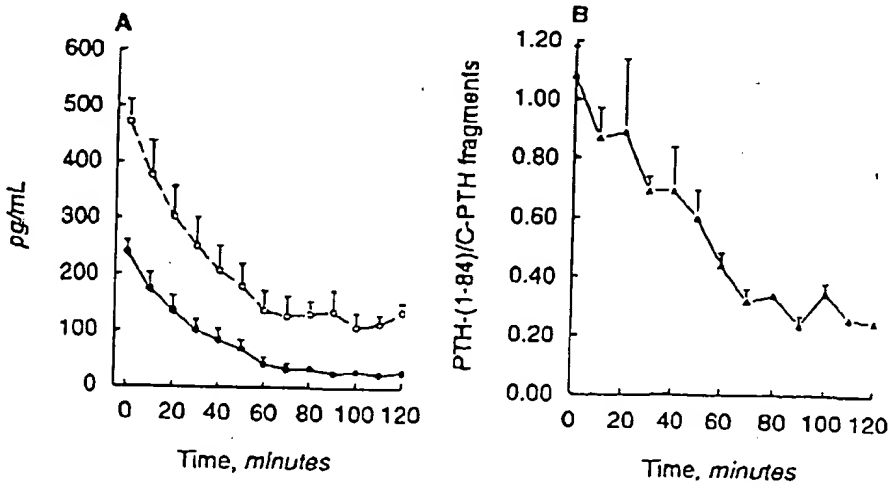

Fig. 7. Changes in PTH peptides during a two-hour calcium gluconate infusion in six patients on dialysis. (*A*) Plasma PTH-(1-84) (●) and intact PTH (○). (*B*) PTH-(1-84)/C-PTH fragment ratio.

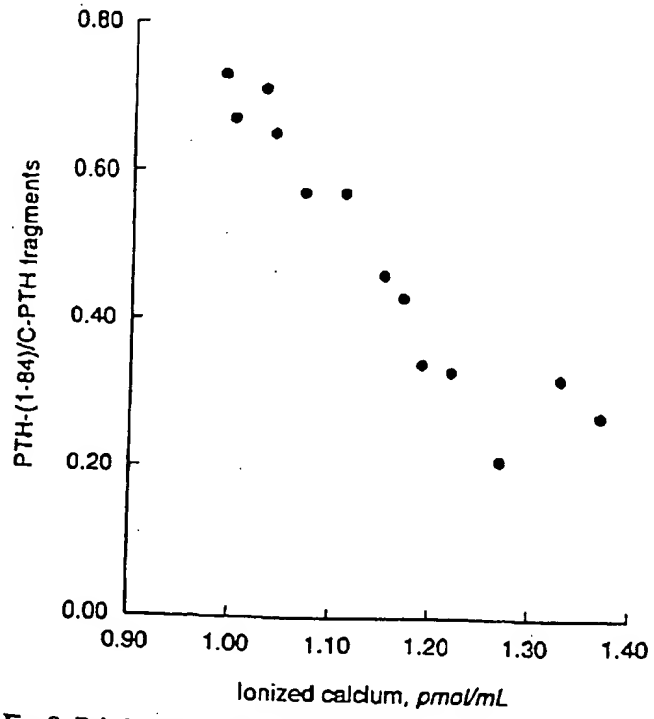

Fig. 8. Relationship between ionized calcium levels and PTH-(1-84)/C-PTH fragments ratio in 8 patients on dialysis during calcium gluconate infusion.

C-PTH fragments is associated with increased bone turnover, whereas predominance of C-PTH fragments over PTH-(1-84) is most often associated with low bone turnover. The present data lend support to the notion of an antagonistic effect of the C-PTH fragments on PTH-(1-84) action on bone, and extend preclinical observations [24, 32–36] to the dialysis population. The wide range of the obtained results in the PTH-(1-84)/C-PTH fragment ratio (from 0.01 to 14.2) demonstrates that a simple fixed percentage of intact PTH cannot be identified for prediction of the large C-PTH fragments. Even though it is likely that the calculated C-PTH fragments contains several fragments, one can assume that 7-84 PTH is the major antagonist since it was shown to be by far the most potent antagonist among all tested PTH fragments [25]. The remaining scatter between the PTH-(1-84)/C-PTH fragment ratio and bone turnover could be related to different target organ responses at the receptor or post-receptor levels to given levels of PTH.

The mechanism(s) by which the large C-PTH fragments may antagonize the effect of PTH-(1-84) on bone is unclear. It has been speculated that this large amino-terminally truncated fragment (most likely 7-84 PTH) may act as competitive antagonist or regulate the expression or sensitivity of the PTH receptors [11]. Although the 7-34 PTH fragment (which is part of 7-84 PTH) has a binding domain to PTH-1R [48, 49], it is still a poor competitor of 1-34 PTH (which is the active sequence of PTH) for the PTH-1R [32, 33]. This finding renders it unlikely that the antagonistic action of 7-84 PTH on bone effects of PTH-(1-84) is mediated through the PTH-1R. Evidence is accumulating for the expression of functional C-terminal receptors from studies in a variety of skeletally derived cells [13, 18, 25–31]. The basic residues Arg25 and Lys 53 have been recently reported to be critical for effective interaction of PTH-(1-84) with receptors for C-terminal portions of PTH-(1-84) [31]. Further studies are needed to elucidate the precise mode of action of the large C-PTH fragments on bone, in particular if the C-PTH fragments interacts with the PTH-1R, the C-terminal PTH receptor, or another receptor.

Another clinically relevant question concerns the factors that regulate the relative production of PTH-(1-84) and large C-PTH fragments by parathyroid glands. Prevailing serum calcium levels at time of blood sampling did not have an impact on the diagnostic value of the different PTH peptides. This might be due to the cross-sectional design of the study and more certainly to the various degrees of hyperplasia and/or calcium sensitivity of the parathyroid glands in the studied patients ex-

plaining that comparable calcium levels are often observed at different PTH levels [50]. However, our present study demonstrates that changes in serum calcium act as regulators of the relative amount of circulating PTH-(1-84) and C-PTH fragments. These data extend the results of a recent study that demonstrated that plasma levels of PTH-(1-84) decreased more readily than intact PTH with calcium infusion [11]. This is also in agreement with previous in vitro and in vivo studies that showed that bovine parathyroid glands incubated in hypocalcemic medium almost exclusively secrete PTH-(1-84), whereas exposure to hypercalcemic medium results in mostly degraded PTH [51–58]. Taken together, these data suggest that in ESRD patients receiving high doses of calcium on a daily basis, the balance between PTH-(1-84) and C-PTH fragments might favor the synthesis and secretion of the C-PTH fragments, which may result in blunting of PTH-(1-84) actions on bone. It is also of note that replacement of aluminum-containing phosphate binders by calcium salts in the middle 1980s coincided with the emergence of low turnover bone disease without aluminum [59–61]. Further studies are needed to elucidate whether calcitriol or other medications and/or coexistent disease states also have a direct or indirect effect on the PTH-(1-84)/C-PTH fragment ratio.

The present data indicate that interpretation of results of hormone radioimmunoassays may be substantially improved as more bioactive peptides are identified. The level of the measured hormone may reflect not only the entire hormone molecule but also hormone fragments, which may have antagonistic or synergistic effects on the parent molecule. Precedent for this concept is seen with prolactin, which generates a 16 kD N-terminal fragment in mammary tissue or ventral prostate that is then recirculated [62, 63]. This prolactin fragment has its own receptor [64] and has an opposite effect on angiogenesis than prolactin [65]. This, as for PTH, would constitute a useful compensatory mechanism to avoid overactivity of the active hormone.

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